Virus Nucleic Acids Formed in Chick Embryo Cells Infected with Semliki Forest Virus

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SUMMARY

The three species of virus RNA (45s, 26s and 20s) formed during infection of chick embryo cells with Semliki Forest virus were isolated by sequential extraction of the cells with phenol and then with phenol + sodium dodecyl sulphate. They were then separated either by sucrose density gradient centrifugation, or by polyacrylamide gel electrophoresis. The 20s RNA was relatively resistant to the action of ribonuclease, in contrast to the single-stranded 26s and 45s species. The 20s RNA was the first species to be formed in infected cells followed by 26s and then by 45s RNA. An attempt to demonstrate the intermediate role of 20s RNA in virus RNA synthesis by the displacement of radioactive by non-radioactive uridine ('pulse-chase' experiment) was unsuccessful, but both 26s and 45s RNA displaced radioactivity from 20s RNA on heating above the thermal transition temperature and slow cooling. Comparison of the sedimentation velocities and electrophoretic mobilities of the 26s and 45s RNA species, together with the sharp thermal transition at 65° of 45s RNA to 26s RNA suggested that 45s RNA consisted of two 26s RNA species, and that the different sedimentation characteristics did not indicate different conformations of the same molecule.

INTRODUCTION

Infection of chick embryo cells with arboviruses leads to the formation of three species of RNA. These are a single-stranded species sedimenting at 45s which is the RNA of the virus particle, a single-stranded species sedimenting at 26s termed ‘interjacent RNA’ (Martin, 1967) of unknown function and a double-stranded species sedimenting at 20s which is relatively resistant to the action of ribonuclease, and probably functions as an intermediate in virus RNA synthesis. This double-stranded species has a high thermal transition temperature (Sonnabend, Martin & Mecs, 1967), and the base ratio analysis was similar to that predicted for a duplex containing one strand of 45s (or 26s) RNA and a second complementary strand (Friedman & Berezesky, 1967; Friedman, 1968). It was the first species of RNA formed, as determined either by labelling early in infection (Friedman, 1968) or by using short pulses of radioisotopes (Friedman, Levy & Carter, 1966), and it was the principal product of virus-specified polymerase enzyme in vitro (Martin & Sonnabend, 1967). Thus, a considerable body of evidence points to its intermediate role in RNA synthesis although there has been no direct demonstration of this. The 45s RNA is very sensitive to the action of ribonuclease and is therefore single-stranded. It does, however,

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possess some secondary structure since the RNA found in western equine encepha-
lo-myelitis virus has a thermal transition temperature of 57.5°C (Sreevalsen et al. 1968).
The role of 26s RNA is unknown: it was the first single-stranded species to be de-
tected by the use of short pulses of radioisotopes (Friedman et al. 1966) and it has the
same base composition as 45s RNA (Friedman & Berezovsky, 1967; Sreevalsan et al.
1968). It can be formed from 45s RNA by heating to 90°C, dialysis against water or
centrifugation in the presence of 8 M-urea or 50% dimethylsulphoxide, and there was
some conversion of 26 to 45s by dialysis against 0.5 M-NaCl or 0.05 M-sodium acetate,
PH 4.0 (Sreevalsan et al. 1968). These authors were unable to separate 26s RNA from
45s RNA on columns of methylated albumin plus kieselguhr, and concluded that 26s
was a different conformational form of 45s RNA and not a portion of that RNA.

Studies on the role of 26s RNA have been hindered by the difficulty of separating
it from 20s RNA by sucrose density gradient analysis, for only partial resolution is
obtained even after prolonged centrifugation (Friedman et al. 1966). Precipitation of
single-stranded RNA with lithium chloride (Sreevalsan et al. 1968) has been used to
prepare 26s RNA, but no studies have yet been reported on the properties of 20s
RNA isolated without the use of ribonuclease. In this paper we report two methods
for the complete quantitative resolution of the virus nucleic acids, and the use of these
methods to study the role of 20s and 26s RNA in virus replication. A brief report of
some of these results has already been published (Cartwright & Burke, 1969).

METHODS

Materials. Actinomycin D was given by Merck, Sharp & Dohme Ltd; [5-3H]uridine
(27.6 Ci/m-mole) and [2-14C]uridine (53 mCi/m-mole) were obtained from the Radio-
chemical Centre, Amersham, Bucks. Sodium dodecyl sulphate (specially pure) was
obtained from British Drug Houses, Ltd, Poole, Dorset. Ether and phenol were
freshly redistilled before use.

Media, cells and viruses were described by Walters, Burke & Skehel (1967).

Extraction of total virus nucleic acids. Chick cell monolayer cultures (approximately
10 x 10^6 cells/culture) were infected with Semliki Forest virus (10 p.f.u./cell) for 1 hr
at 36°C, the cultures washed twice and overlaid with 10 ml. of maintenance medium
containing 0.5 µg./ml. of actinomycin D. [3H]uridine (50 µCi/culture) was added at a
suitable time after infection (normally 4 hr) and the cells incubated for 1 hr at 360
before washing twice with ice-cold phosphate-buffered saline. The cells were scraped
off the glass, sodium dodecyl sulphate was added to a concentration of 0.5% and the
RNA extracted by vigorous shaking with water-saturated phenol for 10 min. at room
temperature. After centrifugation, the aqueous layer was again extracted with phenol,
and the residual phenol removed from the final aqueous phase by extracting with
ether. Excess ether was removed in a stream of nitrogen.

Sequential extraction of nucleic acids. Cells were infected, washed and labelled with
[3H]uridine as before. After the washed cells had been scraped from the glass the RNA
was extracted with phenol at 4°C, and the aqueous phase was washed twice with phenol
and three times with ether, before the ether was removed with a stream of nitrogen.
The phenol layer and the interphase were washed twice with phosphate-buffered saline
and then phosphate-buffered saline containing 0.5% sodium dodecyl sulphate was
added and the mixture shaken vigorously for 10 min. at room temperature. The second
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extract was washed twice with phenol and then three times with ether before removal of the ether as before.

Extraction of cellular RNA. Chick cell cultures were incubated with [14C]uridine (5 µCi/culture of 120 x 10^6 cells) for 12 to 18 hr at 36° before extraction of the cellular RNA with phenol and sodium dodecyl sulphate.

Fractionation of virus RNA on sucrose gradients. Samples of RNA (1 ml.) were layered on to 25 ml. linear sucrose gradients (5 to 20 % sucrose, 0.1 M-acetate, pH 5.0) and centrifuged at 4° for 16 hr at 22,500 rev./min. in the SW25 rotor of the Spinco L2 preparative ultracentrifuge. After centrifugation 1 ml. samples were collected either by puncturing the tubes or by use of an Isco density gradient fractionator. Radioactivity of the fractions was determined as described by Skehel & Burke (1968).

Fractionation of virus RNA on polyacrylamide gels. This was done as described by Peacock & Dingman (1967) using 2.5 % acrylamide, except that NNN'N-tetramethyl-ethylene diamine (0.075 %) was used instead of dimethylaminopropionitrile. The gels (0.5 x 8 cm.), in 'Perspex' tubes, were subjected to electrophoresis for 30 min. at 150 V before application of the sample in 0.1 M-sodium acetate buffer (pH 5.0) containing 20 % sucrose and further electrophoresis for 1 hr at 4° at 150 V and 3 mA/gel. The gels were frozen, cut into sections 2 mm. thick, solubilized and the radioactivity determined as described by Hay, Skehel & Burke (1968). Ribosomal RNA, labelled with [14C]-uridine, was added as a marker to both sucrose gradients and polyacrylamide gels. The radioactivity due to 3H and 14C was determined by a channel ratio method (Hendler, 1964), with the aid of a punch-tape accessory and computer programme written by Mr J. Drewry of this department.

Treatment with ribonuclease. Preliminary experiments were undertaken to determine conditions which just caused complete degradation of single-stranded RNA. The procedure finally adopted was treatment with ribonuclease (1 µg./ml.) for 10 min. in 0.3 M-NaCl and 0.03 M-sodium citrate [2 x standard saline citrate (2 x SSC)] at 37°. The RNA was precipitated, filtered, washed and the radioactivity determined as described by Skehel & Burke (1968).

Determination of thermal transition temperature of RNA. Samples were dialysed for 24 hr against 2 x SSC before heating portions (0.2 ml.) in sealed ‘Pyrex’ glass tubes in an oil bath to the appropriate temperatures. They were kept at these temperatures for 10 min. and were then plunged into a mixture of solid CO2 + ethanol. After being thawed the samples were treated with ribonuclease as above, before precipitation, filtering, washing and determination of radioactivity.

Hybridization procedure. After dialysis overnight against 2 x SSC, the samples (0.3 ml.) were then either left untreated, or heated at 115° for 10 min. and allowed to cool slowly overnight in an oil bath. After this heat treatment, samples were either incubated with ribonuclease as above or left untreated. The results shown are the mean of quadruplicate determinations.

Determination of buoyant density in caesium sulphate. RNA in 0.001 M-EDTA buffered with 0.01 M-tris + HCl, pH 7.7, was mixed with caesium sulphate in the same buffer and adjusted to \( \eta_{26} = 1.3815 \) (density 1.60 g./cm³ 1fft, Voet & Vinograd, 1961). The mixture was centrifuged at 35,000 rev./min. for 60 hr at 20°, and fractions collected from the bottom of the tube.

Precipitation of RNA. RNA in phosphate-buffered saline was precipitated by the addition of 2 vol. ethanol and left at -10° overnight. Single-stranded RNA was
precipitated by the addition of an equal volume of 4 M-LiCl and left for 48 hr at 4°C.

**Brief labelling of virus RNA.** Cells were infected, washed and medium containing actinomycin D (0.5 pg./ml.) added as described above. Four hours after infection 50 μc of [3H]uridine was added the plates incubated for 3, 15 or 60 min. before washing thrice with ice-cold phosphate-buffered saline. The total nucleic acids were extracted by the phenol + sodium dodecyl sulphate method. Equal amounts of ribosomal RNA, labelled with [14C]uridine, were added to each sample in order to determine the efficiency of extraction of the virus RNA.

**Labelling of virus RNA with 1-hr pulses throughout infection.** The cells were incubated with actinomycin D (0.5 μg./ml.) for 2 hr before infection, washing and incubation as described above. [3H]uridine (50 μc) was added for 1 hr periods at 0, 1, 2, 3, 4½ and 6 hr after infection. The RNA was extracted by the sequential extraction procedure, and separated by sucrose density gradient centrifugation. Ribosomal RNA, labelled with [14C]uridine, was added before each extraction so that samples of virus RNA could be quantitatively compared. The amount of the various species of RNA was determined by computation of the areas of the peaks obtained on sucrose gradient analysis.

**'Pulse-chase' experiments.** Cells were infected, washed and incubated with medium containing actinomycin D as described above. Three and a quarter hours after infection 10 μc of [3H]uridine was added per culture, and after 15 min. the medium was removed, the cell sheets washed five times with maintenance medium and then incubated with medium containing 0.5 μg./ml. of actinomycin D and 10⁻⁵ M-uridine. RNA was extracted by the sequential procedure at 0, 90, 150, 180 and 210 min. after addition of the non-radioactive uridine and the extracted RNA separated on sucrose gradients. Ribosomal RNA, labelled with [14C]uridine, was added before each extraction, and the amount of the various species of RNA determined as described above.

**RESULTS**

**Separation and Extraction of virus RNA**

When chick embryo cells which had been infected with Semliki Forest virus were treated with actinomycin D and then labelled with [3H]uridine before extraction of the nucleic acids with phenol and sodium dodecyl sulphate, three species of RNA were obtained. These were the 45s single-stranded species found in the virus particle, the 26s single-stranded species found in infected cells and the 20s 'ribonuclease-resistant' species, all of which have been observed by others (Friedman et al. 1966; Sonnabend et al. 1967). These species were only partially resolved by sucrose density gradient centrifugation but were readily resolved by polyacrylamide gel electrophoresis (Fig. 1). The evidence leading to identification of the peaks obtained by polyacrylamide gel electrophoresis is described later.

When the infected cells were extracted with phenol, only 20s RNA was extracted, and subsequent extraction with phenol + sodium dodecyl sulphate extracted the 45s and 26s species, as shown by sucrose density gradient analysis (Fig. 2) or polyacrylamide gel electrophoresis (Fig. 3). This sequential extraction provided a method for the separation of the 20s and 26s RNA species, and their properties form the subject of this paper.
Properties of 20s RNA

The double-stranded RNA found in arbovirus-infected cells has been widely regarded as completely resistant to the action of ribonuclease (Friedman et al. 1966; Sonnabend et al. 1967), and the product of the virus induced polymerase in vitro has been shown to be degraded by less than 20% after incubation with 2.5 μg of the enzyme/ml for 90 min. (Martin & Sonnabend, 1967). Indeed, the relative proportions

![Graph](image)

Fig. 1. Polyacrylamide gel electrophoresis of virus nucleic acids. Infected cells were labelled with [3H]uridine before extraction with phenol + sodium dodecyl sulphate. Ribosomal RNA, labelled with [14C]uridine, was added as marker before electrophoresis. Virus RNA labelled with [3H]uridine (---●---●), ribosomal RNA labelled with [14C] (---○---○).

| Table 1. Effect of treatment of virus RNA species with ribonuclease. |
|-------------------------------|-------------------|-------------------|-------------------|
| TCA-precipitable material (as % of original counts) | 20s RNA | 26s RNA | 45s RNA |
| Salt concentration relative to that of standard saline citrate | Ribonuclease concentration (μg./ml.) | 5 min. incubation | 10 min. incubation | 30 min. incubation | 5 min. incubation | 10 min. incubation | 30 min. incubation | 5 min. incubation | 10 min. incubation | 30 min. incubation |
| 2 | 0.01 | 90 | 77 | 69 | 32 | 12 | 5 | 33 | 12 | 4 |
| 1 | 0.01 | 78 | 66 | 61 | 25 | 8 | 3 | 21 | 8 | 3 |
| 0.5 | 0.01 | 68 | 53 | 47 | 17 | 7 | 3 | 12 | 4 | 1 |
| 2 | 0.1 | 85 | 73 | 66 | 20 | 9 | 4 | 19 | 4 | 1 |
| 1 | 0.1 | 76 | 66 | 56 | 13 | 7 | 3 | 10 | 2 | 1 |
| 0.5 | 0.1 | 60 | 47 | 43 | 8 | 4 | 2 | 8 | 1 | 1 |
| 2 | 1.0 | 68 | 59 | 49 | 11 | — | — | 12 | 1 | — |
| 1 | 1.0 | 59 | 48 | 39 | 9 | — | — | 4 | — | — |
| 0.5 | 1.0 | 46 | 38 | 31 | 7 | — | — | 4 | — | — |
| 2 | 10.0 | 55 | 45 | 38 | 8 | 3 | 4 | — | — | — |
| 1 | 10.0 | 45 | 34 | 27 | 6 | — | — | — | — | — |
| 0.5 | 10.0 | 38 | 25 | 24 | 4 | — | — | — | — | — |

The three samples of RNA used had 2900 counts/min. (20s RNA); 2500 counts/min. (26s RNA) and 2400 counts/min. (45s RNA). They were prepared by sequential extraction of infected cells followed by sucrose density gradient centrifugation. Peak fractions were pooled and dialysed against 0.5 × SSC to remove sucrose.
of 20s and 26s RNA in a mixture have been estimated by assuming that 20s RNA is 100% ribonuclease resistant, and measuring the effect of enzyme action on the mixture (Mecs et al. 1967). However, the measurements made on 20s RNA isolated from infected cells were made on material which was always partly contaminated with 26s RNA, which is itself extremely sensitive to ribonuclease. We therefore measured

![Graph](image)

Fig. 2. Sucrose density gradient analysis of the phenol (above) and the phenol + sodium dodecyl sulphate (below) extracts of infected cells labelled with [3H]uridine. Fractions were analysed for acid precipitable radioactivity before (---●---) and after (○—○) treatment with 1 μg/ml. of ribonuclease for 10 min. at 37° in 2 x SSC. The arrows show the position of the ribosomal markers.

the ribonuclease sensitivity of 20s material isolated by phenol extraction. First, conditions were chosen that gave complete degradation of single-stranded 26s RNA with the minimum effect on 20s RNA (Table 1). Using optimum conditions (namely 1 μg/ml. for 10 min. at 37° in 2 x SSC), the ribonuclease sensitivity of the 20s peak obtained after a sucrose density gradient centrifugation was measured. It was only about 50% resistant to the action of ribonuclease (Fig. 2). This figure was obtained on many occasions. Some of the ribonuclease sensitivity could have been due to the presence of radioactive cellular 28s ribosomal RNA (which is completely extracted by phenol) (Walters et al. 1967), or virus 26s RNA, although the percentage breakdown
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caused by ribonuclease action was remarkably constant throughout the peak. Ribosomal RNA contamination was ruled out by labelling actinomycin-treated uninfected cells with a similar amount of [3H]uridine, and centrifuging on a sucrose gradient the RNA extracted by phenol; negligible radioactivity was found in the 20 to 26s position. However, it was possible that some 26s virus RNA was extracted by the phenol, and polyacrylamide gel electrophoresis of the phenol extracted RNA (Fig. 3) also suggested this. Therefore, this extract was further purified by precipitation of the single-stranded RNA by 2 M-lithium chloride. Some radioactive RNA was precipitated along with the cold carrier RNA, but sucrose density gradient centrifugal analysis showed that,

![Fig. 3. Polyacrylamide gel electrophoresis of the phenol (above) and the phenol + sodium dodecyl sulphate (below) extracts of infected chick cells labelled with [3H]uridine. The arrows show the position of the ribosomal markers.](image)

although it apparently contained some 26s RNA, the major component had a sedimentation coefficient of about 22s and was still about 35% resistant to the action of ribonuclease (Fig. 4). Thus some partially ribonuclease-resistant material was precipitated with the ribosomal RNA. The ribonuclease-resistance of the RNA which was not precipitated by lithium chloride rose to 75% but was not increased by further treatment with lithium chloride. The material prepared in this way gave a single peak on polyacrylamide gel electrophoresis, and mobility was slightly increased by incubation with ribonuclease before gel electrophoresis (Fig. 5). The material had a density of 1.645 g./cm. 3 in caesium sulphate and a thermal transition temperature of 107°C in 2 x SSC. After treatment with ribonuclease (1 μg./ml. for 10 min. at 37°C in 2 x SSC) the density in caesium sulphate fell to 1.630 g./cm. 3, again suggesting the partially single-stranded character of the 20s material. Martin & Sonnabend (1967) found a
Fig. 4. Sucrose density gradient analysis of (A), the precipitate obtained by lithium-chloride treatment of the phenol extract and (B), the corresponding supernatant fluid. Fractions were assayed for acid-precipitable radioactivity before (●●) and after (○○) ribonuclease treatment. The arrows show the position of the ribosomal markers.

Fig. 5. Polyacrylamide gel electrophoresis of (A). The supernatant fluid obtained by lithium-chloride treatment of the phenol extract. (B) fraction no. 15 from the sucrose density gradient analysis of the phenol extract (see Fig. 2). (C). The supernatant fluid obtained by lithium-chloride treatment of (B), and (D), the product obtained by ribonuclease treatment (1 µg./ml. for 10 min at 37° in 2 x SSC of B).
density of 1.615 to 1.625 g./cm$^3$ for the 20s material extracted from infected cells, but inspection of their figure shows that the peak is asymmetrical and that some material with a density of higher than 1.640 g./cm$^3$ is present. Martin & Sonnabend (1967) found the T$_m$ of the 20s material to be 103° in 1 x SSC, while Friedman & Berezesky (1967) found a value of 97° in 0.02 M KCl. Thus it was very unlikely that the definite but limited ribonuclease sensitivity of the 20s RNA was due to the presence of single-stranded RNA, and we conclude that it was probably due to the protruding tails of single-stranded RNA attached to a single complementary strand of RNA. Examination by polyacrylamide gel electrophoresis of the product obtained by heating 20s RNA to 115° showed that it contained material of about 15s plus some material of about 4s (Fig. 6). The amounts of the latter material which were formed varied somewhat from experiment to experiment and may reflect slight differences in conditions, but in all experiments the material of approximately 15s was the major component.

![Fig. 6. Polyacrylamide gel electrophoresis of 20s RNA before (above) and after (below) heating to 115° followed by rapid cooling.](image)

Friedman (1968) reported that he could not recover intact single-stranded RNA by heat denaturation of virus RNA extracted from infected cells early in infection; this RNA was probably a mixture of 26s and 20s RNA. However, treatment with dimethylsulphoxide followed by sucrose density gradient analysis yielded 45s RNA, unchanged 26s RNA, a ribonuclease-resistant peak sedimenting at about 14s and low molecular weight material. The nature of our 15s product is unknown and it is puzzling that the product obtained by heating 45s or 26s RNA (Fig. 8) was not found. At 115° extensive thermal breakdown of the molecules may have been occurring.

**Properties of 26s and 45s RNA**

When infected cells were extracted first with phenol and then with phenol + sodium dodecyl sulphate, the second extract contained 26s and 45s RNA (Fig. 2). There was no detectable 20s double-stranded RNA in this extract as shown by polyacrylamide gel electrophoresis and the complete degradation of all the RNA by very low concentrations of ribonuclease. Furthermore, examination of the supernatant fluid on sucrose
gradients after precipitation of the single-stranded RNA with 2 M-lithium chloride failed to reveal any double-stranded RNA and we concluded that all the double-stranded RNA had been removed by phenol extraction. The two peaks on polyacrylamide gel electrophoresis were identified by separation of 26S and 45S by sucrose density gradient centrifugation followed by gel electrophoresis (Fig. 7). In this way,

the peak at fraction 4 in the gels was identified as 45S RNA, having the same electrophoretic mobility as RNA isolated from the virus particle. The peak at fraction 10 was identified as 26S RNA. Assuming that electrophoretic mobility is governed only by the molecular weight of the RNA (Bishop, Claybrook & Spiegelman, 1967), and taking the molecular weights of chick 28S and 18S RNA to be $1.58 \times 10^6$ and $0.70 \times 10^6$ (Loening, 1968) we calculated that 45 RNA has a molecular weight of $3.4 \times 10^6$ and 26S a molecular weight of $1.74 \times 10^6$. Using the relationship $\text{mol. wt} = 1550 \times \text{s}^{2.1}$ (Spirin, 1963), these values correspond to 39S and 28S respectively.
Both 26s and 45s RNA had the same density as that of ribosomal RNA (1.680 g./cm\(^3\)) as determined by caesium sulphate density gradient centrifugation. Martin & Sonnabend (1967) found a value of 1.680 g./cm\(^3\), while Sreevalsan et al. (1968) found a value of 1.66 g./cm\(^3\) for 45s and 26s RNA isolated from cells infected with western equine encephalomyelitis virus. The latter authors also showed that 45s RNA could be converted to 26s RNA by heating to 90\(^\circ\), or exposure to low ionic strength buffers. We found that 45s RNA from cells infected with Semliki Forest virus was completely converted to 26s RNA by heating above 65\(^\circ\) (Fig. 8). When 45s was heated to 72\(^\circ\) or above, a second species of RNA was produced which, from its mobility in polyacrylamide gels, had a molecular weight of 8.5 x 10\(^5\) and a sedimentation coefficient of 20s. This material was also formed by heating 26s RNA. After heating at 115\(^\circ\) there was some suggestion of breakdown to material of lower molecular weight (about 4s). There was no conversion of 26s to give 45s when material was heated to 90\(^\circ\) and allowed to cool slowly.

Fig. 8. Polyacrylamide gel electrophoresis of 45s and 26s RNA after heating to different temperatures. The nucleic acids were isolated by the sequential extraction procedure followed by sucrose density gradient centrifugation. (1) 45s before heating. (2) 45s heated to 60\(^\circ\) and quickly cooled. (3) 45s heated to 65\(^\circ\) and quickly cooled. (4) 45s heated to 72\(^\circ\) and quickly cooled. (5) 45s RNA heated to 77\(^\circ\) and quickly cooled. (6) 45s RNA heated to 83\(^\circ\) and quickly cooled. (7) 45s RNA heated to 90\(^\circ\) and quickly cooled. (8) 45s RNA heated to 115\(^\circ\) and quickly cooled. (9) 45s RNA heated to 90\(^\circ\) and slowly cooled. (10) 26s RNA heated to 90\(^\circ\) and quickly cooled.
Production of virus nucleic acids in infected cells

When infected cells were labelled with [\(^{3}H\)]uridine for very short times, and the extracted RNA analysed by polyacrylamide gel electrophoresis, it was found (Fig. 9) that both 20s and 26s RNA were labelled but that the former was the more prominent. Only a small amount of 45s RNA (only 5% of the total radioactivity) was found with a 15-min labelling period; it was much more prominent when the labelling period was 1 hr. A similar conclusion was reached by Friedman et al. (1967) as a result of sucrose gradient analysis of the labelled RNA, although they were able to resolve only partially 20 and 26s RNA. This suggested that the first species to be formed was 20s followed by 26s and then 45s RNA. A similar conclusion was reached as a result of analysing the RNA species labelled for 1 hr at different times after infection (Fig. 10). Sequential extraction was followed by sucrose density gradient analysis to enable 20s and 26s RNA to be estimated accurately. The 20s RNA was the main product early in infection but more 26s RNA than 20s RNA was present later. This experiment showed that the radioactivity of the 45s species was a constant proportion of that of the 26s species, suggesting that the conversion of 26s to 45s was the rate limiting
Fig. 10. Production of the three species of RNA at different times after infection. The nucleic acids were extracted by the sequential procedure and analysed by sucrose density gradient analysis.

Fig. 11. Radioactivity of different species of virus RNA and total TCA-insoluble radioactivity after a 15 min. pulse of [3H]uridine and subsequent chase. 20s RNA — ○ — ○ —, 26s RNA — — — — — —, 45s RNA — — — — — —.
reaction in the formation of the latter. A similar conclusion was reached by Sonnabend et al. (1967) as to the relative rates of formation of 20s and 26s RNA, although they made no measurements until 3 hr after infection, and they assumed that 20s RNA was completely resistant to ribonuclease in order to be able to distinguish 26s and 20s RNA. By adding isotope immediately after infection and estimating the radioactivity of the 45s RNA at different times after infection, they showed that 45s RNA was the major product late in infection.

Table 2. Hybridization of 20s RNA with either 26s or 45s RNA

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Heat treatment Ribonuclease treatment</th>
<th>¹C counts/min.</th>
<th>³H counts/min.</th>
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<tr>
<td>20s</td>
<td>+</td>
<td>5571</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1143 (21%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3200 (57%)</td>
<td></td>
</tr>
<tr>
<td>20s</td>
<td>+</td>
<td>2493</td>
<td>14631</td>
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<tr>
<td></td>
<td>+</td>
<td>747 (30%)</td>
<td>2937 (20%)</td>
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<tr>
<td></td>
<td>-</td>
<td>2730</td>
<td>14593</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1632 (60%)</td>
<td>891 (6%)</td>
</tr>
<tr>
<td>20s+26s</td>
<td>+</td>
<td>5490</td>
<td>14302</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1448 (26%)</td>
<td>5566 (39%)</td>
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<td>-</td>
<td>5679</td>
<td>14770</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>3104 (55%)</td>
<td>870 (6%)</td>
</tr>
<tr>
<td>20s+45s</td>
<td>+</td>
<td>2691</td>
<td>14731</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>733 (28%)</td>
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<td></td>
<td>-</td>
<td>1615 (59%)</td>
<td>799 (5%)</td>
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<tr>
<td>20s+45s</td>
<td>+</td>
<td>5553</td>
<td>14700</td>
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<tr>
<td></td>
<td>+</td>
<td>1271 (23%)</td>
<td>5402 (37%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5623</td>
<td>14530</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>3043 (54%)</td>
<td>879 (6%)</td>
</tr>
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</table>

* Figures in parentheses are the per cent of radioactivity resistant to treatment with ribonuclease (1 μg./ml. for 10 min. at 37° in 2 × SSC). The nucleic acids were prepared by sequential extraction of infected cells followed by sucrose density gradient centrifugation. Peak fractions were pooled before dialysis against 2 × SSC.

We attempted to demonstrate the intermediate role of the 20s double-stranded component by means of a 'pulse-chase' experiment. The virus nucleic acids were labelled for 15 min. with [³H]uridine, washed extensively, and the cells were then incubated in the presence of 10⁻⁵ M non-radioactive uridine. At intervals the virus nucleic acids were extracted and separated and the radioactivity of these virus species determined. At zero time the main components of the labelled virus nucleic acids were the 20s and 26s RNA species. There was also more 45s RNA present than usual (about 25% of the total radioactivity), possibly because of the time taken to wash the cells thoroughly before extraction. Over a period of 180 min. the radioactivity of the 20s species decreased slowly while that of 26s and 45s species increased rapidly and then levelled off (Fig. 11). The decrease between 180 and 210 min. may well have been due to a cytopathic effect on the infected cells at nearly 7 hr after infection. Thus,
Although there was a rapid increase in the radioactivity of the 26s and 45s species, there was only a small loss of radioactivity from the 20s species, possibly due to a pool of radioactive uridine in the cells which was not diluted out by the non-radioactive uridine, and it was difficult to draw any conclusion as to the role of the 20s species.

Stronger evidence for the intermediate role of the 20s RNA species was obtained by a hybridization method. When 20s RNA, labelled with $^{14}$C, was heated to $115^\circ$ and allowed to cool slowly, the percentage of radioactivity which was resistant to ribonuclease fell from 57% to 21% (Table 2), suggesting that complete reannealing did not take place. When the 20s RNA was mixed with 26s RNA or 45s RNA, labelled with $^3$H, and then heated and cooled, the percentage of $^{14}$C radioactivity resistant to ribonuclease fell by the same amount, but a considerable percentage of the $^3$H radioactivity became resistant to ribonuclease. When the amount of 20s RNA was doubled, the amount of 26s RNA which became resistant to ribonuclease also doubled. Since there was no self-annealing of either 26s or 45s RNA, either in the absence (Table 2) or in the presence (Table 3) of ribosomal RNA, the results show that both 26s and 45s RNA contain nucleotide sequences complementary to those of 20s RNA, as would be expected if 20s was an intermediate in virus RNA biosynthesis.

Table 3. Lack of hybridization of 26s RNA and 45s RNA with cellular RNA

<table>
<thead>
<tr>
<th>RNA Species</th>
<th>Heat treatment at 115°C</th>
<th>Ribonuclease treatment</th>
<th>$^{14}$C counts/min.</th>
<th>$^3$H counts/min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal RNA</td>
<td>+</td>
<td>+</td>
<td>14003</td>
<td></td>
</tr>
<tr>
<td>26s + Ribosomal RNA</td>
<td>+</td>
<td>+</td>
<td>4974 (4%)*</td>
<td>10723 (7%)</td>
</tr>
<tr>
<td>20s + Ribosomal RNA</td>
<td>--</td>
<td>+</td>
<td>14209</td>
<td></td>
</tr>
<tr>
<td>45s + Ribosomal RNA</td>
<td>--</td>
<td>+</td>
<td>4493 (3%)</td>
<td></td>
</tr>
</tbody>
</table>

* See Table 2.

**DISCUSSION**

The three species of virus RNA can be resolved, either by sequential extraction followed by sucrose density gradient centrifugation, or by use of the superior resolving power of polyacrylamide gel electrophoresis. The separation of 20s from 26s and 45s RNA by the sequential extraction procedure probably depends on the association of most of the single-stranded RNA with either the virus particle or its precursors, for both the virus particle and the 140s nucleoid particle contain 45s RNA and the 65s particle found in infected cells contain 26s RNA (Friedman & Berezesky, 1967; Yin & Lockart, 1968), whereas the 20s RNA, although associated with the polymerase, would not be enclosed in a protein or lipoprotein envelope. Virus RNA cannot be extracted from eastern equine encephalitis virus by cold phenol alone (Wecker, 1959) nor can the rapidly labelled nuclear RNA of chick-embryo cells, although ribosomal RNA is extracted completely (Walters et al., 1967). The phenol extract was 50 to 60% resistant
to the action of ribonuclease. It was not contaminated with radioactive ribosomal RNA, and precipitation with lithium chloride yielded a supernatant fluid which was about 75 % resistant to ribonuclease action and which gave a symmetrical peak on sucrose density gradient analysis (Fig. 4B) or on polyacrylamide gel electrophoresis (Fig. 5A). However, examination of the lithium chloride precipitate on a sucrose density gradient (Fig. 5B) showed that the material was only 35 % resistant to ribonuclease and that there was a partially resolved 26s peak. Thus the phenol extract contained a small amount of free 26s RNA which may have been newly synthesized RNA not yet associated with virus protein structures. It also contains a partially double-stranded structure which was insoluble in lithium chloride, probably because of the partially single-stranded nature of this material. This material is similar to that described by Plagemann & Swim (1968) as one of the products of the mengo virus RNA polymerase in vitro and thought by them to be a replicative intermediate.

Further purification of the lithium chloride supernatant fluid did not increase the percentage of ribonuclease resistance above 75 %, suggesting that this material was still partially single-stranded. The relative sensitivity of this material may be contrasted with that of the virus-specified polymerase product in vitro (Martin & Sonnabend, 1967) which was degraded by about 2 % under the same conditions, suggesting that this latter structure is a replicative form. The identification of the phenol-extractable material as a replicative intermediate was further strengthened by the change in density in caesium sulphate density gradient centrifugation away from that of single-stranded RNA on treatment with ribonuclease. Friedman (1968) claimed to have isolated a replicative intermediate from chick-embryo cells infected with Semliki Forest virus by extracting with phenol + sodium dodecyl sulphate just over 1 hr after infection. However, this procedure also extracts 26s RNA which is detectable by this time (Fig. 4B) and it is likely that his early labelled RNA consisted of a mixture of 2os and 26s RNA, which was not resolved by sucrose density gradient analysis. If the phenol-extractable material is a replicative intermediate, then it should be possible to demonstrate transfer of radioactivity to 26s or 45s RNA. A number of attempts were unsuccessful, possibly because the pool of intracellular uridine did not equilibrate with that in the medium. Similar unsuccessful attempts to chase radioactive uridine in tissue culture cells have been recorded before (Warner et al. 1966). However both ³H-labelled 26s and 45s displaced radioactivity from ¹⁴C-labelled 20s RNA on heating to 115 ° and cooling, showing that the 20s RNA contained negative strands. Thus, the phenol-extractable material has many of the properties of the replicative intermediate, although whether the production of a relatively ribonuclease-resistant structure is a consequence of phenol extraction is not known.

It is remarkable that the 20s ribonuclease-resistant RNA migrates more rapidly than either 45s or 26s RNA on electrophoresis in polyacrylamide gels, since the replicative form and the replicative intermediate of bacteriophage Qβ and of poliovirus move more slowly than the corresponding virus RNA (Pace, Bishop & Spiegelman, 1967; Noble, Kass & Levintow, 1969). We have no explanation of this result and further work is in progress.

Addition of radioisotopes early in infection and the use of short labelling periods showed that 26s RNA was the first single-stranded species to be formed, 45s RNA being labelled much more slowly. This suggested that 26s RNA played an intermediate role in the formation of 45s RNA, a suggestion strengthened by the interconversion
of 45s and 26s RNA described by Sreevalsan et al. (1968). We have shown that the thermal conversion of 45s RNA to 26s RNA takes place over a narrow temperature range between 60 and 65°, and since the thermal transition temperature of the RNA of a related virus, western equine encephalomyelitis, is 57.5° (Sreevalsan et al. 1968), it may well be that the conversion of 45s to 26s is associated with breaking of hydrogen bonds, either to give a molecule of the same size but different conformation or a molecule of lower molecular weight. Sreevalsan et al. favoured the former alternative, suggesting that the lower sedimentation coefficient of 26s RNA was due to the presence of a more open structure. However if this was so then 26s should move more slowly than 45s on polyacrylamide gel electrophoresis, whereas the reverse is true. We therefore suggest that 26s RNA is a smaller molecule than 45s, thus explaining its lower sedimentation coefficient and greater electrophoretic mobility. If mobility in polyacrylamide gels is governed solely by the molecular weight of the RNA, then 45s RNA has a molecular weight of 3.4 x 10⁶ and 26s, 1.74 x 10⁶, suggesting that 45s RNA comprises two molecules of 26s RNA. On further heating 26s is converted to a molecule with a calculated molecular weight of 8.5 x 10⁵, suggesting that 26s RNA comprises two such molecules. This breakdown product would have a sedimentation coefficient of 20s (Spirin, 1963) and no single-stranded RNA of this size has been detected in infected cells. It remains to be seen whether this species is involved in virus RNA synthesis, but it is remarkable that a virus RNA with a molecular weight of only 3.4 x 10⁶ should be made up of two, and possibly four, subunits. Since Sreevalsan et al. (1968) claimed that 26s RNA is infectious, the coding potential of this virus is carried by a RNA of molecular weight 1.7 x 10⁶, and this may explain why the virus contains only two proteins (Hay et al. 1968) in contrast to the larger number of proteins found in the picornaviruses. It is of interest that the RNA of Rous sarcoma virus has recently been shown to be disassociated by dimethylsulphoxide or heat to give probably four subunits (Duesberg, 1968; Montagnier, Goldé & Vigier, 1968).

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REFERENCES


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