The Nucleic Acid of Sendai Virus and Ribonucleic Acid Synthesis in Cells Infected by Sendai Virus

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SUMMARY

RNA was extracted from purified Sendai virus, labelled with $^{32}$P, and sedimented in sucrose density gradients. Under conditions of high ionic strength (0.1 M-NaCl) the sedimentation coefficient was 57 S. In the presence of 0.005 M-EDTA, the sedimentation coefficient was reduced to 40 S, indicating that the RNA of the virus exists as a single-stranded molecule.

The intracellular synthesis of virus-directed RNA during the latent period of infection of chick embryo cells by Sendai virus in the presence of actinomycin was also studied. Cells were extracted by the phenol + sodium dodecyl sulphate method at 2, 4, 6 and 7 hr after infection, having previously been exposed to $[^{3}H]$uridine for at least 2 hr. It was found that the first newly formed RNA had a sedimentation coefficient of 18 S and could be detected between 2 and 4 hr after infection. From 4 hr onwards a 57 S component was found in increasing amounts until 7 hr after infection. Substantially similar results were obtained when infected cells were treated with detergent alone, and the mixture immediately centrifuged on density gradients except that the principal RNA components now had sedimentation values of 57 S and 23 S.

INTRODUCTION

This paper describes an attempt to isolate and characterize the RNA from Sendai virus, and to follow the formation of virus-directed RNA in infected chick embryo cells. Sendai virus is very similar biologically to Newcastle disease virus, except that it has a considerably longer latent period (Barry, 1965). Our findings are substantially similar to those of Duesberg & Robinson (1965) and Bratt & Robinson (1967) for Newcastle disease virus, but in infected cells we observed the sequential formation of virus-directed RNA of only two types. An 18 to 23 S RNA appears about 2 hr after infection and accumulates, to be followed about 2 hr later by the accumulation of 57 S RNA.

METHODS

Materials. $[^{5},^{3}]$H$|$uridine (50 mc/μmole) and $^{32}$P (orthophosphate in solution containing 1 mg./ml. phosphate buffer pH 7.0) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Actinomycin D was a gift from Merck, Sharp and Dohme, Rahway, New Jersey, U.S.A. Bovine pancreatic ribonuclease was purchased from the Worthington Biochemical Corporation. Purified

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tobacco mosaic virus was a gift from Mr M. Rees of the Agricultural Research Council Virus Research Unit, Huntingdon Road, Cambridge.

**Virus.** Sendai virus strain 960 was used throughout and consisted of allantoic fluid preparations harvested 72 hr after inoculation of ten-day-old chick embryos with $10^5$ EID 50 of infected allantoic fluid and subsequent incubation at $37^\circ$. Stocks were stored frozen at $-70^\circ$. Virus was labelled with $^{32}$P by inoculating each of a dozen 10-day-old embryos with 0.2 ml. of phosphate buffer (pH 7.0) containing 0.5 mc of $^{32}$P as orthophosphate, 4 hr before infection.

**Purification of virus.** Approximately 100 ml. of infected allantoic fluid was obtained from eggs inoculated with $^{32}$P and the virus was purified as follows. After removal of cell debris by centrifugation for 10 min. at 3000 rev./min. (2500 g) in a Mistral 6 L centrifuge, the virus was deposited by centrifugation of the supernatant fluid for 1 hr at 21,000 rev./min. in a Spinco 21 rotor. The pellet was resuspended in 5 ml. of phosphate buffered saline, treated for 10 sec. in a blender then shaken with an equal volume of cold fluorocarbon (Arcton II3, Imperial Chemical Industries) for 30 sec. and finally the phases were separated by brief centrifugation (Kingsbury, 1966). The fluorocarbon treatment had no appreciable effect on the haemagglutinin titre of the virus. The virus concentrate was then layered on to a 60 to 15% sucrose gradient and centrifuged at 20,000 rev./min. for 4 hr at $10^\circ$ in an SW 25 swinging bucket rotor. This treatment produced a pearly white pellet of virus on the bottom of the tube and several prominent bands of non-viral allantoic fluid material at different levels of the gradient. The pellet, consisting of 80,000 agglutinating doses of virus, was resuspended in 90 ml. of buffer containing 0.01 M-tris+HCl (pH 7.4), 0.001 M-EDTA and 0.1 M-NaCl and deposited by centrifugation at 30,000 rev./min. for 1 hr in the Spinco 30 rotor. Finally, the pellet was resuspended in 5 ml. of the same solution, and the RNA extracted.

**Cell culture.** Primary cultures of chick embryo cells were prepared from 10-day-old embryos, and seeded at a concentration of $10^6$ cells/ml. into either 4 oz prescription bottles (10 ml./bottle) or Roux bottles (50 ml./bottle). The growth medium was medium 199 (Burroughs Wellcome Ltd) supplemented with 10% calf serum. The cells were incubated at $37^\circ$ for 48 hr before use, by which time they had formed a confluent monolayer and were either infected with Sendai virus or used as controls. Cells were infected by replacing the medium in each bottle with growth medium containing a $10^{-1}$ dilution of stock virus and actinomycin at a concentration of 2 $\mu$g./ml. Prescription bottles were inoculated with a total volume of 1 ml. and Roux bottles with 10 ml. This inoculum was estimated to provide a multiplicity of about 100 infectious units per cell. All containers were shaken gently at room temperature for 30 min. to allow adsorption to occur and then the inoculum was removed and replaced by fresh 199 medium containing 2 $\mu$g./ml. actinomycin. Two $\mu$g/ml of $[^{3}H]$uridine were added to the medium 2 hr before the conclusion of the experiment.

**Isolation of nucleic acid.** RNA was extracted from purified Sendai virus to which had been added 0.3 ml. (2 mg.) of purified TMV, in the manner described previously for influenza virus (Davies & Barry, 1966).

To obtain RNA from virus-infected cells, two isolation methods were tested. The first was described by Bratt & Robinson (1967). Cell monolayers were washed with 0.01 M-tris+NaCl buffer (pH 8.5) containing 0.1 M-NaCl and 0.001 M-EDTA. Nucleic acid was obtained from the cells by addition of the same buffer containing 1% sodium dodecyl sulphate and 0.5% 2-mercaptoethanol. After shaking gently for
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5 to 10 min. the cell + detergent mixture was extracted 3 times with buffer-saturated phenol and the nucleic acid in the aqueous phase was precipitated twice with ethanol.

The second method was described by Prince & Adams (1966). Cells were taken up in a small volume of 0.5% sodium dodecyl sulphate in acetate buffer pH 5.1, and layered directly on to sucrose density gradients.

Density-gradient centrifugation. Except where otherwise stated, 0.2 ml. samples of 3P-labelled RNA from purified virus were layered on to 5 to 20% linear sucrose gradients, total volume 5.2 ml., prepared in 0.01 M-acetate buffer (pH 5.3), containing 0.1 M-NaCl and 0.001 M-EDTA and centrifuged for 210 min. at 32,000 rev./min in a Spinco SW 39 rotor. RNA extracted from cells was layered on to similar gradients prepared in buffer containing 0.01 M-tris+HCl (pH 7.4), 0.1 M-NaCl and 0.001 M-EDTA, and centrifuged at 35,000 rev./min. for 210 min. in the SW 39 rotor. Ten-drop fractions were collected from the bottom of the tubes with a micrometer syringe sampling device.

Characterization of RNA. With RNA preparations prepared by the phenol + detergent method, each sample from the density gradients was collected in a flat bottomed tube (5 ml. capacity); 0.3 ml. of distilled water was added to each sample, and the optical density at 260 mμ determined in silica micro-cells in a Unicam SP 500 Spectrophotometer. To determine radioactivity, 3.5 ml. of Bray's fluid (Bray, 1966) was added to each tube and after thorough mixing, the samples were counted in a Packard Tricarb liquid scintillation counter, series 4000.

For RNA obtained by the method of Prince & Adams (1966), samples were collected by needle puncture on to Whatman no. 2 filter-paper rectangles which were then placed in cold 10% trichloracetic acid and kept for 1 hr at 4°. The filter papers were washed three times with 5% trichloracetic acid then with an ether + ethanol mixture (3:1), and finally with two changes of ether. After drying, the specimens were placed in flat-bottomed vials with 3.5 ml. of a dioxane-based scintillation fluid, and counted in the scintillation counter.

RESULTS

The sedimentation characteristics of Sendai-virus RNA

The sedimentation pattern of 3P-labelled Sendai-virus RNA was compared with that of TMV RNA determined by optical density (Fig. 1). The S value of TMV RNA was 31 S (Davies & Barry, 1966). By comparison with the TMV RNA peak the sedimentation constant of the rapidly sedimenting 3P-labelled RNA peak was estimated to be 57 S, assuming that the distance travelled from the meniscus was proportional to the sedimentation coefficient (Martin & Ames, 1961). There was a small peak of radioactivity in the position of the TMV peak, but this was not a consistent finding (see Fig. 2a) and probably represented aggregates of degraded RNA. This gradient contained a total of 52,892 counts/min. of which 19,104 counts/min. (approximately 36%) constituted the rapidly sedimenting peak. By analogy with other large RNA-containing viruses (Robinson, Pitkanen & Rubin, 1965; Duesberg & Robinson, 1965), the 57 S peak was assumed to represent unbroken virus RNA molecules. Treatment of the labelled RNA for 10 min. at room temperature with 0.1 μg./ml. ribonuclease completely degraded the RNA so that all radioactivity remained at the top of the density gradient after centrifugation.

Although Sendai RNA is ribonuclease-sensitive, the effect of salt concentration on
its sedimentation characteristics was tested because it has been claimed that this RNA occurs naturally as a double-stranded molecule (Tikchonenko et al. 1964). Two sucrose density gradients were prepared, one containing 0.1 M-NaCl + 0.001 M-EDTA, while the other contained 0.005 M-EDTA alone. A single batch of $^{32}$P-labelled Sendai virus RNA was divided between the gradients and they were centrifuged simultaneously (Fig. 2). The distribution of RNA in the gradient prepared in acetate buffer containing 0.001 M-EDTA + 0.1 M-NaCl (Fig. 2a) was very similar to that in Fig. 1, except that a slightly higher value (61 S) was calculated for the virus RNA, and there was extensive aggregation of the TMV RNA in the lower half of the gradient. However, the sedimentation coefficient of both Sendai RNA and TMV RNA was markedly reduced (values of 40 S and 25 S respectively) in the gradient containing 0.005 M-EDTA (Fig. 2b) and there was much less aggregation of the TMV RNA at the bottom of the gradient. These findings suggest that the RNA of Sendai virus is single stranded.

Fig. 1. Sucrose gradient analysis of RNA extracted from purified $^{32}$P-labelled Sendai virus. Fraction 1 was from the bottom of the tube. The arrow indicates the optical density peak of the TMV RNA.

The formation of RNA in chick embryo cells infected with Sendai virus

In NDV-infected cells, at least four viral-specific RNA components were found in the presence of actinomycin, those occurring in the largest amounts being 18 S, 22 S and 35 S (Bratt & Robinson, 1967). We thought it likely that some or all of these components were degraded forms of 57 S virus RNA. We therefore looked for relative differences between various types of newly formed RNA in cells infected by Sendai virus, using the methods of Bratt & Robinson (1967), and compared the findings with those obtained by the simpler extraction procedure described by Prince & Adams (1966). Confluent, primary cultures of chick embryo cells were infected with
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an estimated multiplicity of 100 infectious units per cell. At the time of infection, actinomycin (2 μg/ml) was added to the cultures and was present throughout the experiment. Under these conditions there was a latent period of approximately 6 hr before newly formed virus was released. To determine when virus-directed RNA made its appearance, [3H]uridine (2 μCi/ml) was added to the medium at various times after infection and 2 hr later the RNA was extracted. On several occasions the [3H]uridine was present for a total period of 4 hr. Controls consisted of batches of uninfected cells treated with actinomycin and handled in exactly the same way as virus-infected cells.

![Fig. 2. The effect of ionic strength on the sedimentation rate of 32P-labelled Sendai virus RNA (a) in the presence of 0.01 M-EDTA + 0.1 M-NaCl and (b) in the presence of 0.005 M-EDTA. O - - - O, optical density (E260); — radioactivity.](image)

Using the extraction method described by Bratt & Robinson (1967), cells were extracted 2, 4, 6 and 7 hr after infection, and the results are presented in Fig. 3a, b, 4a, b. In the control cells, incorporation of [3H]uridine was invariably confined to 4S RNA. In infected cells no evidence of virus-directed RNA synthesis was detected earlier than 2 hr after infection. Between 2 and 4 hr (Fig. 3a) the first formed RNA sedimented as a distinct peak coinciding with the 18S ribosomal RNA of the cells. There was also a slight shoulder of activity in the 28S region. During the next 2 hr interval (Fig. 3b) the 18S peak increased and a new, distinct peak appeared at 57S. During the period between 2 and 6 hr after infection (Fig. 4a) a distinct 18S peak and a smaller 57S peak were detected. These findings suggested that the 18S component was made first, about 2 hr after infection, and that 2 hr later 57S RNA, assumed to be virus RNA, began to appear. Between 5 and 7 hr after infection (Fig. 4b) the relative proportions of the two types of RNA changed considerably. At this time the 57S RNA was present in similar amounts to the 18S RNA. The RNA extracted from 32P-labelled whole virus sedimented in exactly the same position in the gradient as the 57S [3H]uridine labelled RNA extracted from Sendai-infected cells. This gradient also
contained TMV RNA (position indicated with an arrow), and there was a small $^{3}P$[H]uridine peak in the same position, which may have been due to aggregation. The RNA extracted from cells between 2 and 6 hr after infection was hydrolysed by ribonuclease (Fig. 5).

Fig. 3. The formation of RNA in chick-embryo cells between 2 and 6 hr after infection with Sendai virus. (a) Between 2 and 4 hr; (b) between 4 and 6 hr. ○ --- ○, Optical density (E260); ● --- ●, RNA labelled with $[H]$uridine (counts/min.).

Fig. 4. The formation of RNA in chick embryo cells between 2 and 7 hr after infection with Sendai virus. (a) Between 2 and 6 hr; ○ --- ○, optical density (E260); ● --- ●, RNA labelled with $[H]$uridine (counts/min.). (b) Between 5 and 7 hr, $[H]$uridine present between 5 and 7 hr after infection. A sample of virus RNA ($[3]P$-labelled Sendai RNA + TMV RNA) was added to the cellular RNA before centrifugation of the 5 to 20 % sucrose gradient. The position of optical density peak for the TMV RNA is indicated by the arrow. △ --- △, $[3]P$-labelled virus RNA; ● --- ●, cellular RNA labelled with $[H]$uridine (counts/min.).

When cells were disrupted with sodium dodecyl sulphate alone and immediately placed on gradients (Prince & Adams, 1966) similar results to those described above were found (Fig. 6a, b). Once again there were two main components. Assuming the
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leading peak to be 57 S the second component, although heterogeneous, had a peak at 23 S. Furthermore, the relative proportions of each component at different times after infection resembled those found with the phenol + detergent method (Fig. 4a, b).

Fig. 5. The effect of RNase on the RNA formed between 2 and 6 hr after infection of chick embryo cells with Sendai virus. A sample of RNA from the experiment shown in Fig. 4a was treated with 0.1 µg/ml RNase for 10 min. at room temperature and then subjected to density gradient analysis. ○---○, optical density (E260); ●--●, [3H]uridine (counts/min.)

DISCUSSION

We found that a considerable proportion of 32P-labelled Sendai-virus RNA occurred as a homogeneous component with a sedimentation constant of 57 S when subjected to velocity sedimentation in sucrose density gradients containing 0.001 M-EDTA and 0.1 M-NaCl. Duesberg & Robinson (1965) demonstrated that Newcastle-
disease-virus RNA contained a component thought to be intact virus RNA which, when centrifuged in gradients containing 0.001 M-EDTA and 0.1 M-NaCl, had a sedimentation constant of 57 S. Thus, the nucleic acids of both viruses sedimented identically in sucrose gradients of similar ionic strength.

The sedimentation rate of single-stranded RNA molecules is strongly influenced by salt concentration (Boedtker, 1960; Robinson et al. 1965). Thus, when Newcastle disease virus RNA was centrifuged in 0.05 M-NaCl, the sedimentation coefficient was reduced to 49.2 S (Kingsbury, 1966), and similarly when Sendai virus RNA was centrifuged in 0.05 M-KCl, the sedimentation coefficient was 42 S (Iwai et al. 1966). We found that the absence of salt reduced the sedimentation coefficient of Sendai virus RNA to 40 S. From this we conclude that Sendai virus RNA, like Newcastle disease virus and TMV RNA, is a single-stranded molecule and not a rigid, double-stranded molecule, as has been claimed by Tikchonenko et al. (1964).

The long latent period characteristic of Sendai virus replication is an asset in the study of the intracellular development of virus-directed RNA synthesis. Throughout this period it has been possible to discern the sequential formation of two types of RNA, as characterized by their sedimentation coefficients. In this respect, these studies provide additional information to that reported by Bratt & Robinson (1967), who confine their attention to RNA synthesis occurring only between 6 and 8 hr after infection of chick embryo cells with Newcastle disease virus, a time when virus release is nearing completion, and comparatively much later than the times studied here. The sequence of events found when cellular RNA was extracted by the method of either Bratt & Robinson (1967) or Prince & Adams (1966) appeared to be as follows. The earliest RNA formed was 18 to 23 S, the exact S value depending mainly on the method of extraction used. This species of RNA appeared 2 hr after infection and was produced at a constant rate until at least 7 hr after infection. This component probably represents the ‘early’ RNA synthesis, previously reported in cells infected with myxoviruses (Bukrinskaya et al. 1966). Four hr after infection, 57 S RNA appeared and progressively accumulated until, by 7 hr after infection, it was present in similar amounts to the 18 to 23 S components. The simplest conclusion to be drawn from these findings is that only two major species of newly synthesized RNA occur in cells infected with Sendai virus i.e., 57 S virus RNA and 18 to 23 S, presumably messenger, RNA. However, inspection of all gradients indicates that considerable levels of radioactivity exist between the two major components and that the 23 S component seen in Fig. 6a, b is very heterogeneous. This intermediate, polydisperse activity may have been due entirely to non-specific aggregation of RNA formed by clumps of intact RNA or RNA broken down during extraction. Throughout these studies and those reported in the preceding paper (Barry & Davies, 1968) we have constantly encountered varying degrees of RNA aggregation apparently due to the presence of salt in the gradients, and we think that this factor must be considered when attempting to characterize different types of RNA according to their sedimentation behaviour. These considerations are also pertinent to the work of Bratt & Robinson (1967), who favour the idea that at least four virus-specific components, designated 57, 35, 22 and 18 S, exist in cells infected with Newcastle disease virus. Unlike ourselves, they found very little 57 S RNA in their cells, so that the 35 S and 22 S material may have contained degraded 57 S material.

It is necessary, however, to consider the alternative possibility that, in addition to
the major components, various intermediate species of RNA exist in cells infected with Sendai virus. Unfortunately, these studies provide no information on this point, but it is clear that since the RNA made between 2 and 6 hr after infection was completely susceptible to ribonuclease, none of the intermediate radioactivity found between the 57 S and 18 S components was due to the presence of a double-stranded replicating form.

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REFERENCES


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