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Fate of Sendai Virus Ribonucleoprotein in Virus-infected Cells

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The cytoplasmic extracts of Ehrlich ascites tumor cells infected with $^{32}PO_4$ and ^{3}H-leucine-labeled Sendai virus have been examined during the course of infection with respect to sedimentation behavior and buoyant densities of input virus radioactivity. It was found that ^{32}P and ^{3}H radioactivities were coincident, and, at 30 min after infection, the bulk of radioactivity was recovered in the polysome region of a sucrose gradient in the position of Sendai virus ribonucleoprotein (210S). The heterogeneity of radioactivity profiles appeared at 1 hr after infection and increased during 6 hr of incubation. The buoyant densities of input virus components were determined by banding in CsCl gradient. Here again the bulk of coincident ^{32}P and ^{3}H radioactivity at 30 min after infection banded at the same density as Sendai virus ribonucleoprotein (1.31 g/cm^3.) This component disappeared at 3 hr after infection, and ^{32}P and ^{3}H radioactivities were now found in components banded at densities 1.38, 1.41, 1.45, 1.49, and 1.55 g/cm^3. The results presented are consistent with the idea that virus ribonucleoprotein is retained in the cytoplasm of infected cells during at least 6 hr of incubation, being partly deproteinized in the course of infection. The nature of components which banded at ρ = 1.41, 1.45, 1.49, and 1.55 as complexes of partly deproteinized ribonucleoprotein with ribosomes will be described in a separate paper.

There are two different points of view concerning the way in which myxoviruses enter cells. According to some investigators, the viral particles gain entry to the host cell by a process of viropexis (5, 14, 16). However, electron microscopic investigations of influenza and Sendai virus penetration into cells by Morgan et al. (12, 13) gave evidence that the virions undergo destruction on the cell membranes, and, as a result of disruption of viral and cell membranes, virus ribonucleoprotein (RNP) is released into cytoplasm. These data are entirely consistent with the hypothesis of Hoyle (8) and Hoyle and Finter (9).

So far no information has appeared concerning the properties of penetrated virus components and their alterations in infected cells other than the data of Hoyle and Finter (9) that the ribonucleoprotein of influenza virus is hydrolyzed in the cell with release of amino acids and free nucleic acid.

We made an attempt to follow the fate of Sendai virus ribonucleoprotein in infected Ehrlich ascites tumor cells. The synthesis of virus-specific components and formation of infectious RNP have been described in this system (3). The data presented show that about 50% of penetrated parental ribonucleic acid (RNA) exists in the cytoplasm in the form of virus ribonucleoprotein which is partly deproteinized in the course of infection.

MATERIALS AND METHODS

Materials. Actinomycin D was obtained from Merck, & Co., Inc., Rahway, N. J. Uridine-5-^{3}H(208 mc/millimole), $^{32}PO_4$, and ^{3}H-leucine (5mc/millimole) were obtained from the Radioisotope Centre, Leningrad.

Virus. Methods for propagating Sendai virus strain 960 and for purifying unlabeled and labeled virus were described previously (1). Virus was labeled with $^{32}PO_4$, ^{3}H-uridine, and ^{3}H-leucine by inoculating 0.4, 0.25, and 0.2 mc of isotopes, respectively, in 10-day-old chicken embryos simultaneously with the virus inoculum. The final virus preparation contained 10^{12} to 10^{13} EID_{50}/ml and 25,000 to 50,000 hemagglutinating-activity units (HA)/ml.

When the purity of the virus preparation was determined by the test of virus adsorbtion on chicken red cells, 90 to 95% of the radioactivity was found to be associated with virions.

Cells. Mice were inoculated intraperitoneally with 4×10^7 hyperdiploid Ehrlich ascites tumor cells. Cells obtained on the 7th day after tumor inoculation were washed with Earle saline, suspended in Hanks solution without glucose, prepared with 0.01 M

tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, buffer (*p*H 7.4), and supplemented with 0.2% glutamine (6) so that the final concentration was 2×10^7 cells/ml; the cells were incubated at 37 C in suspension cultures. Actinomycin D (2 μg/ml) was added to the medium 1 hr before virus infection. Purified labeled virus was added to the medium to provide a multiplicity of about 500 infectious units per cell. More than 95% of the virus was shown to be adsorbed on the cells 30 min after infection.

Cell fractionation and sucrose density gradient centrifugation. At different intervals postinfection, samples of cells were removed, and the cells were quickly chilled, washed with cold Earle saline and then with reticulocyte standard buffer [RSB, 0.01 M Tris-hydrochloride (*p*H 7.4), 0.01 M NaCl, 0.0015 M magnesium acetate], resuspended in 2 ml of RSB with 0.01% macaloid, and allowed to stand at 0 C for 10 min. The cells were disrupted by 15 to 20 strokes of a Dounce homogenizer. Unbroken cells, cell debris, and nuclei were sedimented at 1,200 × *g* for 10 min. When the cells incubated for 30 min with ^{3}H-thymidine were fractionated in this way, 97% of the trichloroacetic acid insoluble, tritium-containing material was found in the nuclear pellet and 3% in the cytoplasmic extract, indicating that the supernatant fluid was relatively free of nuclear materials.

The pellet (1,200 × *g*) was resuspended in 0.44 M sucrose. Then the nuclei were washed with 0.25 M sucrose and centrifuged through 2.0 M sucrose in a Superspeed 50 centrifuge in bucket-rotor (3 × 5) at 35,000 rev/min for 30 min. The pellet containing purified nuclei was used for counting parental virus radioactivity. The supernatant fluid (1,200 × *g*) was centrifuged at 10,000 × *g* for 10 min, and this supernatant fluid (cytoplasmic extract) in a volume of 2 ml was layered on a 17-ml linear 17 to 40% sucrose gradient prepared in RSB with 0.01% of macaloid and centrifuged in a Superspeed 50 centrifuge rotor (3 × 20) at 25,000 rev/min for 3 hr at 2 C. The fractions were collected from the bottom of the tubes by means of a density gradient fractionator attached to a continuously recording ultraviolet light analyzer at 260 nm (LKB, Sweden).

Trichloroacetic acid-precipitable radioactive material was determined by addition of 0.1% caseine to each sample, followed by trichloroacetic acid to make a 5% solution. The resulting precipitates were collected and washed with 5% trichloroacetic acid on membrane filters (Millipore Corp., Bedford, Mass.) which were dried, placed in scintillation vials with 10 ml of toluene scintillation fluid, and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Centrifugation of cytoplasmic extracts in preformed CsCl density gradient. The cells were broken in 0.01 M phosphate buffer (*p*H 7.4) containing 0.001 M magnesium acetate, and the cytoplasmic extracts were made 8% with respect to formaldehyde by the addition of appropriate volumes of 40% formaldehyde buffered at *p*H 6.8 (17). After 24 hr of fixation at 4 C the samples were mixed with "light" solutions of CsCl. Two kinds of preformed 4.5-ml density gradients of CsCl solutions were made with ρ = 1.32 to 1.72 and 1.1 to 1.5 g/cm^3. The gradients contained a constant concentration of phosphate buffer (0.003 M), Mg (0.003 M), and formaldehyde (8%). They were centrifuged at 35,000 rev/min in the bucket-rotor (3 × 5) for 15 hr at 8 C and fractionated by puncturing the bottoms of the tubes and collecting 16-drop fractions. The absorbance at 260 nm was determined, and refractive indexes in two drops from fractions 3 and 4 were measured and related to densities (10). Then the fractions were precipited with cold 5% trichloroacetic acid collected on membrane filters, (Millipore) and radioactivity was counted as described earlier.

RESULTS

Sedimentation patterns and buoyant density of Sendai virus RNP. Sendai virus labeled with $^{32}PO_4$ or with $^{32}PO_4$ simultaneously with ^{3}H-leucine was treated with 0.5% sodium deoxycholate (DOC) to release RNP and analyzed on density gradients of sucrose (17 to 40%) and CsCl (ρ = 1.1 to 1.5 g/cm^3). When DOC-treated ^{32}P-labeled virus was analyzed on sucrose gradients, the bulk of radioactivity was recovered in fractions 5 and 6 from the bottom of the tube (Fig. 1a).

In some experiments, DOC-treated virions were mixed before centrifugation with cellular extracts obtained from uninfected Ehrlich tumor cells, and similar sedimention patterns were obtained (Fig. 1b). With ribosomes as a marker (80*S*), the sedimentation coefficient of the radioactivity peak was estimated as 210*S*. This value corresponds to the *S* value of RNP isolated from Newcastle disease virus (11). The faster sedimenting components were also observed in RNP preparations of Sendai and Newcastle disease viruses

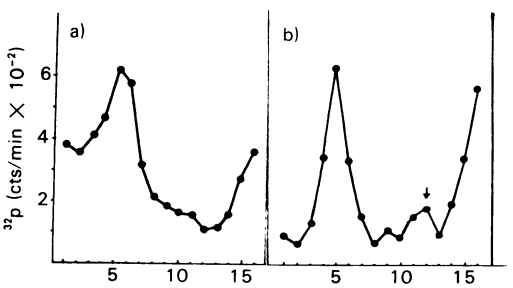

FIG. 1. *Distribution of ^{32}P after sucrose gradient centrifugation of DOC-treated ^{32}P-labeled Sendai virions (a), and DOC-treated virions mixed with cytoplasmic extract from uninfected cells (b). ^{32}P-labeled Sendai virions were treated with 0.5% DOC and disrupted virions layered on sucrose gradient and centrifuged as described. Cytoplasmic extract from uninfected cells was obtained as described in Materials and Methods and mixed with disrupted virions before centrifugation. The arrow in Fig. 1b and other figures shows the position of monosomes as revealed by OD.*

and have been suggested to represent an aggregated viral nucleocapsid (7, 11).

When DOC-treated virus labeled simultaneously with $^{32}PO_4$ and ^{3}H-leucine was banded in a CsCl-density gradient, the profiles of ^{32}P and ^{3}H radioactivity coincided, and the radioactivity was found in two bands with densities 1.22 and 1.31 g/cm^3 (Fig. 2b). Untreated virus gave a single band at about ρ = 1.22 g/cm^3 (Fig. 2a). Thus the density band at ρ = 1.22 appears to represent an unmodified virus, and a smaller band at density of ρ = 1.31 appears to represent virus ribonucleoprotein. The same value for Sendai virus ribonucleoprotein was found by Hosaka (7).

Nature of parental virus radioactivity in the cytoplasmic fraction. Actinomycin-treated cells were infected with ^{3}H-uridine-labeled virus, and the distribution of radioactivity was determined in cell fractions 1 hr after infection; the results of three experiments are shown in Table 1. It may be seen that the radioactivity in the cytoplasm composes 40 to 48% of total radioactivity.

To determine which virus components are responsible for the radioactivity found in the cytoplasm, cells were infected with the virus labeled simultaneously with $^{32}PO_4$ and ^{3}H-leucine, and cytoplasmic extracts obtained 1 hr after infection were centrifuged in a sucrose density gradient. It is seen from Fig. 3 that the profiles of ^{32}P and ^{3}H radioactivities are coincident, and the radioactivity is discovered in nearly the same position in which virus RNP was found to sediment. This experiment suggests that virus RNP is present in cytoplasmic extracts of infected cells 1 hr after infection.

The alterations of sedimentation patterns of intracellular virus RNP in the course of infection are shown in Fig. 4. At 30 min after cell infection with ^{32}P-labeled virus, the homogenous peak of radioactivity in fractions four and five from the bottom of the tube (sedimentation coefficient of about 210*S*) and the smaller components (60-110*S*) are seen. At 1 hr after infection, the radioactivity is distributed in a more disperse way, and a shoulder is observed around fractions 4 and 5, whereas the main portion of label is found in fraction 6 (sedimentation coefficient 180*S*). At 3 and 6 hr after infection, the heterogeneity of radioactivity profiles increases. A certain amount of radioactivity is usually observed at the top of the gradient at all intervals after infection.

Similar alterations of radioactivity profiles in the course of infection were observed when the cells were infected with ^{3}H-leucine-labeled virus (Fig. 5).

To follow in greater detail the alterations of radioactivity profiles, 32 fractions, instead of 16, were collected from the gradient in one experiment. Two peaks of radioactivity which appeared as early as 30 min after infection and decrease in the faster sedimenting component 1 hr after infection were detected in this case (Fig. 6).

Thus a component present in cells at 30 min after infection sedimented as virus RNP, and its sedimentation coefficient decreased during the course of infection. The minor components of $^{32}PO_4$- and ^{3}H-leucine-labeled input virus radioactivity sedimenting in the postribosomal region and at the top of the gradient are difficult to interpret; they may represent partly degraded virus RNP or other labeled virus components

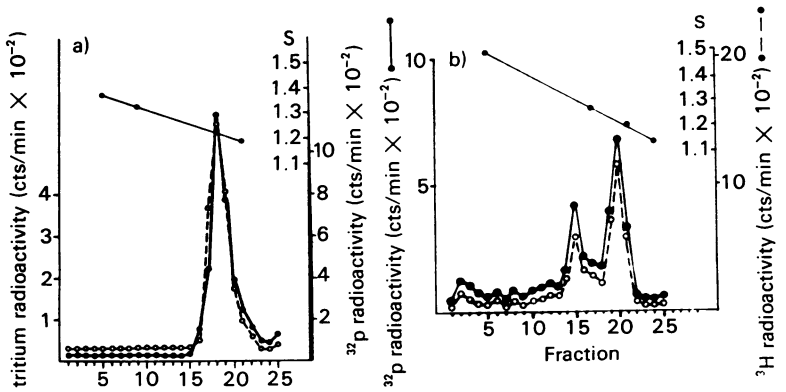

FIG. 2. *Buoyant density distribution of radioactivity after CsCl-density gradient centrifugation of labeled Sendai virus untreated (a) and treated with 0.5% DOC (b). Sendai virus labeled with $^{32}PO_4$ and ^{3}H-leucine and the same virus treated with 0.5% DOC were banded in preformed CsCl-density gradient (ρ = 1.1 to 1.5 g/m^3) for 15 hr at 36,000 rev/min. Fractions were collected and radioactivity counted as described. (●) ^{32}P radioactivity, (○) ^{3}H radioactivity.*

(phospholipids and proteins) that have penetrated into the cells.

Alterations of buoyant densities of intracellular virus RNP in the course of infection. Actinomycin-treated cells were infected with virus labeled simultaneously with $^{32}PO_4$- and ^{3}H-leucine; cytoplasmic extracts obtained 1, 3, and 6 hr after infection were banded in CsCl density gradients.

When cytoplasmic extracts were banded in gradients with ρ = 1.32 to 1.72 g/cm^3, three peaks of optical density (OD) at ρ = 1.55, 1.49, and 1.41 g/cm^3 were observed, which presumably represented, respectively, polysomes and monosomes, small ribosomal subunits, and post-ribosomal particles containing newly synthesized messenger RNA ("informosomes") (2, 15). When the gradient of ρ = 1.1 to 1.5 was used, only the last peak of OD (1.41 g/cm^3) was revealed (Fig. 7).

In cytoplasmic extracts obtained 30 min after infection, part of the ^{32}P and ^{3}H radioactive material was found in a band centered at about ρ = 1.31 g/cm^3; this peak appears to be unmodified RNP penetrated into the cell. In addition, smaller amounts of ^{32}P and ^{3}H banded as a broad peak at about ρ = 1.34 to 1.37, and at ρ = 1.41 g/cm^3.

The lower-density components containing only ^{32}P or only ^{3}H in these gradients apparently represent virus phospholipids and proteins which penetrated into the cell (Fig. 7a), the amounts of these components decreasing 1 hr after infection (Fig. 7b). The peak banded at ρ = 1.31 g/cm^3 significantly diminished at this interval, and the principal peak containing ^{32}P and ^{3}H radioactivity banded at ρ = 1.35 g/cm^3; shoulders around ρ = 1.37 to 1.38 g/cm^3 and a small component at ρ = 1.41 g/cm^3 were also observed. The peak at ρ = 1.31 disappeared at 3 hr after infection, and ^{32}P and ^{3}H radioactivities were found now in five

TABLE 1. *Distribution of radioactivity in cell fractions 1 hr after infection with ^{3}H-uridine labeled virus*

Expt	Counts/min/2 × 10^8 cells	
	Nucleus	Cytoplasm
1	13,679	9,292
2	10,337	8,184
3	3,771	3,444

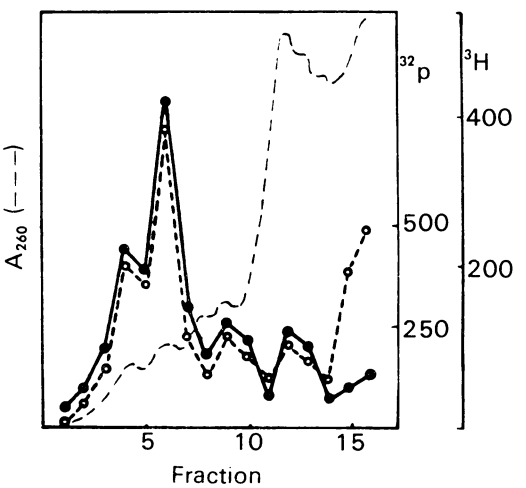

FIG. 3. *Distribution of ^{32}P (●) and ^{3}H (○) after sucrose gradient centrifugation of cytoplasmic extract obtained 1 hr after cell infection with Sendai virus labeled simultaneously with $^{32}PO_4$ and ^{3}H-leucine. Cells (2 × 10^7/ml) were exposed to actinomycin D (2 μg/ml) for 1 hr at 37 C and infected with Sendai virus labeled simultaneously with $^{32}PO_4$ and ^{3}H-leucine (multiplicity of infection 500 EID_{50} postcentrifugation). At 1 hr after infection the cytoplasmic extracts were obtained, layered on a sucrose gradient, and centrifuged. Fractions were collected and analyzed for OD at 260 nm and for acid-insoluble radioactivity. The bottom of the gradient is to the left.*

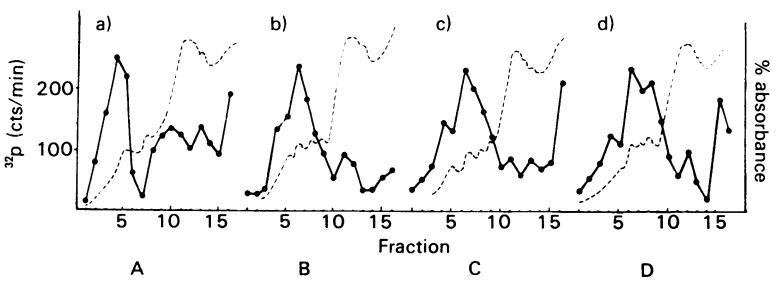

FIG. 4. *Distribution of ^{32}P (●) after sucrose gradient centrifugation of cytoplasmic extracts obtained 30 min (a), 1 hr (b), 3 hr (c), and 6 hr (d) after cell infection with ^{32}P-labeled Sendai virus. Actinomycin-treated cells were infected with ^{32}P-labeled virus; at indicated intervals after infection, the samples of cells were removed and cytoplasmic extracts were prepared and centrifuged.*

components banded at ρ = 1.38, 1.40 to 1.41, 1.45, 1.49, and 1.55 g/cm^3 (Fig. 7c). A picture indistinguishable from this was observed 6 hr after infection (Fig. 7d). The position of radioactive peaks described was repeatedly reproduced in a number of similar experiments. No significant radioactivity which might be due to the free parental RNA was found in pellets.

The ratio of ^{3}H to ^{32}P was different in the components banded at different densities; in the peak banding at ρ = 1.31 g/cm^3 it was the same as in the peak of virus RNP obtained after centrifugation of DOC-treated virus (Fig. 2b); in the components banded at ρ = 1.38 and 1.40 to 1.41 g/cm^3, it was significantly lower. Therefore, when one of the labeled virus preparations was used, ^{3}H radioactivity in the peak of unmodified virus was 46% of ^{32}P radioactivity; in the peak of virus RNP after treatment with DOC, it was 36%. In the peak at ρ = 1.31 g/cm^3 after centrifugation of cytoplasmic extracts of infected cells (30 min after infection), it was 34% and, in the peaks at ρ = 1.38 and 1.41 g/cm^3 (3 hr after infection), it was 19 and 18%, respectively. In the nuclei of the same cells, ^{3}H radioactivity was no more than 6 to 8% of ^{32}P radioactivity.

DISCUSSION

When Ehrlich ascites tumor cells were infected with ^{3}H-uridine-labeled Sendai virus, more than a 50% of input virus radioactivity was recovered

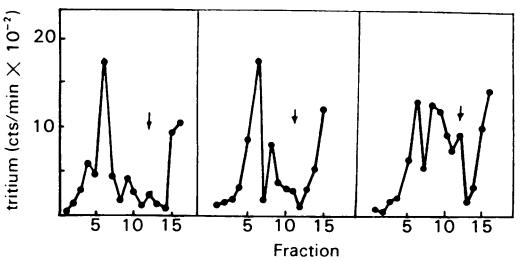

FIG. 5. *Distribution of ^{3}H radioactivity after sucrose gradient centrifugation of cytoplasmic extracts obtained one hour (a), three hours (b), and six hours (c) after cell infection with ^{3}H-leucine labeled virus. The experiments were done as those described in Fig. 4 except that ^{3}H-leucine-labeled virus was applied.*

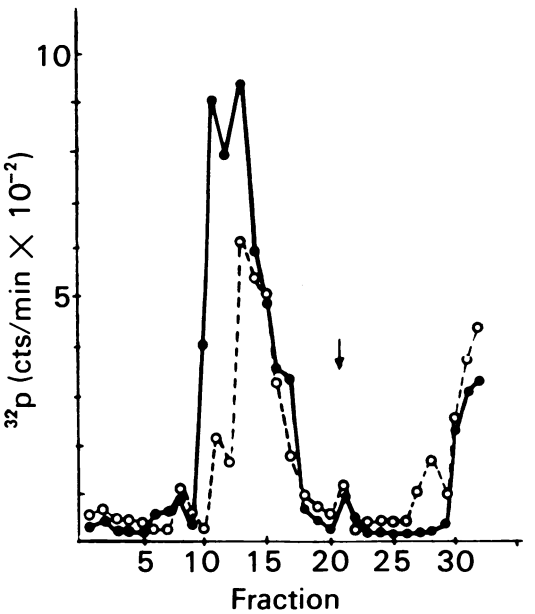

FIG. 6. *Distribution of ^{32}P after sucrose gradient centrifugation of cytoplasmic extracts obtained 30 min postinfection (●) and 1 hr postinfection (○). Experiments were done as were those in Fig. 4, except that 32 fractions instead of 16 were collected.*

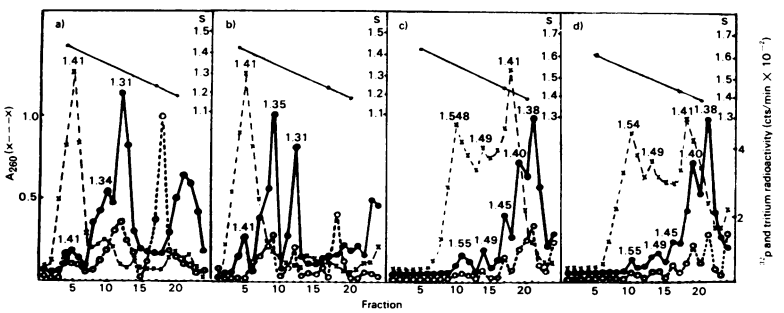

FIG. 7. *Buoyant density distribution of radioactivity after CsCl-density gradient centrifugation of cytoplasmic extracts obtained from cells at different intervals after infection with labeled virus. Actinomycin-treated cells were infected with virus labeled simultaneously with $^{32}PO_4$ and ^{3}H-leucine, and cytoplasmic extracts were obtained (a) 30 min, (b) 1 hr, 3 hr (c), and 6 hr (d) after infection. After 24 hr of fixation with 8% formaldehyde, the cytoplasmic extracts were banded in CsCl-density gradient with ρ = 1.1 to 1.5 g/cm^3 (a, b) and 1.32 to 1.72 g/cm^3 (c, d) at 36,000 rev/min for 15 hr. Symbols: ●, ^{32}P radioactivity, ○, ^{3}H radioactivity.*

in the nuclear fraction at an early stage of infection. These results are in agreement with previously published data on nucleolar localization of Sendai virus parental RNA (4, 17).

A smaller part of input RNA label was found in the cytoplasm. The experiments presented here show that this label is due to virus ribonucleoprotein which is detected as long as 6 hr after infection. During the first hour after infection, its properties (sedimentation coefficient and buoyant density) are indistinguishable from those of RNP obtained after centrifugation of DOC-treated virus. In the course of infection, the intracellular RNP undergoes partial deproteinization and alteration; its sedimentation profiles after centrifugation in sucrose density gradient become more heterogeneous, and the sedimentation coefficient decreases from 210*S* to 180*S* and lower values. Centrifugation in CsCl density gradient showed that the final products appearing at 3 hr after infection are five components banded at ρ = 1.38, 1.41, 1.45, 1.49, and 1.55 g/cm^3. The component of ρ 1.38 appears to represent partly deproteinized RNP, whereas in the other four components the virus RNP is associated with ribosomes (ρ = 1.41, 1.49, and 1.55 g/cm^3) and with ribosomes and virus-induced RNA (ρ = 1.45 g/cm^3; *in preparation*).

About 30 to 40% of input virus radioactivity in the cytoplasm was discovered in pellets obtained after centrifugation at 10,000 × *g*. This radioactivity was shown to be due also to input virus RNP associated with polysomes, because, after EDTA treatment (0.02 M), this radioactivity sedimented in a sucrose gradient into the position of 180 to 210*S*.

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