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Properties of a Ribonucleoprotein Particle Isolated from Nonidet P-40-Treated Rous Sarcoma Virus

NANCY L. DAVIS AND ROLAND R. RUECKERT
Biophysics Laboratory, University of Wisconsin, Madison, Wisconsin 53706

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A ribonucleoprotein particle containing about 20% ribonucleic acid (RNA), and containing little if any phospholipid or glucosamine, was recovered in high yield after treatment of Schmidt-Ruppin strain of Rous sarcoma virus and B77 virus with the nonionic detergent Nonidet P-40. This structure, which probably derives from the internal ribonucleoprotein filament described in electron microscopy studies, contained 80 to 90% of the viral 60 to 70*S* RNA and only about 10% of the protein present in intact virions. It sedimented in glycerol density gradients at approximately 130*S* and had a buoyant density in sucrose of about 1.34 g/ml. Studies with ^{32}P-labeled virus indicated that the ribonucleoprotein particle contained approximately 30 4*S* RNA molecules per 10^7 daltons of high-molecular-weight viral RNA. Intact virions contained about 70 4*S* RNA molecules per 10^7 daltons of high-molecular-weight RNA. Electrophoretic studies in dodecyl sulfate-containing polyacrylamide gels showed that the ribonucleoprotein particle contained only 5 of the 11 polypeptides found in the virion; of these the major component was a polypeptide weighing 14,000 daltons.

Rous sarcoma virus (RSV) is released from the host cell by a budding process. Thus the mature RSV virion consists of a core, containing primarily ribonucleic acid (RNA) and protein, surrounded by a lipoprotein envelope acquired from the plasma membrane of the infected cell. The morphologically defined core includes an intermediate membrane and a central electron-dense nucleoid (7).

Although early attempts to isolate an internal ribonucleoprotein (RNP) substructure from RSV showed it to be relatively unstable (1, 7), conditions have been reported by Coffin and Temin for the isolation of an RNA-containing particle from Nonidet P-40 (NP-40)-disrupted RSV in essentially quantitative yield (5).

Selective degradation of virus envelopes and isolation of internal RNP substructures, coupled with the identification of virion proteins, have been powerful tools for studying the structural organization of other enveloped RNA viruses (23–25). The NP-40 disruption procedure developed by Coffin and Temin for RSV presented the opportunity for initiating a similar biochemical study of the RSV internal structure.

In this communication, we report studies on the products of NP-40 disruption of RSV in which density gradient centrifugation and polyacrylamide gel electrophoresis were used to define an RNP component of RSV in terms of its macromolecular composition, sedimentation coefficient, and buoyant density.

MATERIALS AND METHODS

Source of virus. Secondary cultures of chicken embryo fibroblasts in 60-mm plastic culture dishes, prepared and infected with Schmidt-Ruppin-D strain of RSV (SR-RSV) as described previously (5), were obtained from Howard Temin on day 4 or 5 after infection.

Radioactive labeling of RSV. At 4 or 5 days after infection, modified Eagle culture medium (ET medium) (5) was removed from RSV-infected secondary chicken embryo fibroblast monolayers and replaced by modified ET medium (described below) containing radioactive precursors (obtained from New England Nuclear Corp.) and 2 to 5% fetal bovine serum. After 24 hr of incubation at 37 C, the radioactive virus-containing medium was harvested.

The following labeling media were used: amino acid-free ET medium to which was added 2 to 5% fetal bovine serum and (i) uridine-*G*-^{3}*H* (17.5 μCi/ml, > 2 Ci/mmole) plus L-amino acid-*U*-^{14}*C* mixture (1.75 μCi/ml, 1 mCi/0.67 mg), or (ii) L-amino acid-*U*-^{14}*C* mixture (8.3 μCi/ml), or (iii) L-amino acid-*G*-^{3}*H* mixture (33 μCi/ml, NET-250); complete ET medium containing 2% fetal bovine serum and D-glucosamine-6-^{3}*H*(N) (10 μCi/ml, 1 to 2 Ci/mmole).

RSV doubly labeled with ^{32}P- and ^{3}H-amino acid mixture was prepared by using the following labeling schedule. Infected cultures were incubated at 37 C first overnight in phosphate-free ET medium supplemented with 2% fetal bovine serum; then for the following 24 hr with ^{32}P-orthophosphate (83.5 mCi/ml) in phosphate-free ET medium to which was added 2% fetal bovine serum; and finally for 24 hr with ^{32}P-orthophosphate (83.5 mCi/ml) plus L-amino acid-*G*-^{3}H mixture (8.35 μCi/ml, NET 250) in phosphate-free, amino acid-free ET medium supplemented with 2% fetal bovine serum. Virus was harvested after the first and second 12-hr period of incubation in the mixed labeling medium.

A concentrated suspension of ^{32}P-labeled B77 virus, prepared by John Coffin as described previously (5), was obtained from the laboratory of Temin. This ^{32}P-labeled B77 virus was labeled according to the schedule described above, except that virions labeled between 24 and 48 hr after the addition of ^{32}P to the culture medium were collected in a single 24-hr harvest.

Purification of radioactive virus. Virus was purified using a slight modification of the procedure previously reported (5). All operations were performed at 4 C unless otherwise indicated. Pooled, virus-containing culture fluids were centrifuged for 20 min at 7,000 rev/min in a Sorvall type SS34 rotor. Virus was pelleted from the clarified supernatant by centrifuging either for 50 min at 23,000 rev/min in a Spinco SW25.1 or SW25.2 rotor, or for 1 hr at 25,000 rev/min in a Spinco type 30 rotor. The virus pellets were resuspended in approximately $\frac{1}{50}$ volume of cold ET medium containing 2 to 5% fetal bovine serum by using a rubber policeman. The concentrated virus was sedimented to the interface of a discontinuous sucrose gradient containing 1 ml of 65% (w/v) sucrose and 3 ml of 15% (w/v) sucrose in tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate (Tris-EDTA) buffer (0.01 M Tris-hydrochloride, *p*H 8.1, 0.001 M EDTA) plus 0.1% bovine serum albumin by centrifuging in a Spinco SW65 rotor at 30,000 rev/min for 1 hr. The gradient was fractionated at room temperature through a needle puncturing the bottom of the tube. The interface fractions were pooled, diluted five- to sevenfold with Tris-EDTA buffer, and layered onto a 3-ml linear 15 to 60% (w/v) sucrose gradient containing Tris-EDTA buffer. After centrifugation in a Spinco SW65 rotor for 2 hr at 45,000 rev/min, purified virus was recovered by fractionation as above from a sharp band at 1.14 to 1.16 g/ml, and the pooled virus-containing fractions were frozen at −70 C.

NP-40 disruption and sucrose-D_2O gradients. A suspension of radioactive purified virus representing 0.3 to 10 ml of virus-containing culture medium, with or without the addition of 80 μg of unlabeled purified virus, was adjusted to a concentration of 0.25% (v/v) NP-40 detergent (Shell Chemical Co.) and 1% (w/v) dithiothreitol (DTT, Calbiochem) in Tris-EDTA buffer at 4 C and was layered immediately in the cold onto a chilled linear sucrose gradient made from 30% (w/v) sucrose in Tris-EDTA buffer and 65% (w/v) sucrose in deuterium oxide (D_2O, Sigma Chemical Co.) buffered with 0.01 volume of a Tris-EDTA buffer concentrate (1 M Tris-hydrochloride, *p*H 8.1, 0.1 M EDTA). The gradients also contained 0.4% (w/v) DTT. After centrifugation in a Spinco SW65 rotor at 45,000 rev/min and 4 C for various times, the gradients were collected in 0.15-ml fractions through a needle puncturing the bottom of the tube. Samples of each fraction were counted, and, in some cases, 0.01-ml samples were weighed to determine density.

Equilibrium banding of the NP-40 disruption products was done in similar gradients prepared by using 90% (w/v) sucrose in D_2O, instead of 65% sucrose as above. Gradients were fractionated and analyzed for radioactivity and density in the same way as the 30 to 65% sucrose-D_2O gradients.

Polyacrylamide gel electrophoresis in SDS. Ten percent polyacrylamide gels, cross-linked with 0.3% (v/v) ethylene diacrylate, were prepared and subjected to pre-electrophoresis in electrode buffer containing 3-mercaptopropionate as previously described (14).

Samples of purified RSV in Tris-EDTA-buffered sucrose, RNP in Tris-EDTA-buffered sucrose containing 0.4% (w/v) DTT, and soluble fraction in 0.25% NP-40, 1% DTT, and Tris-EDTA buffer were prepared for gel electrophoresis by adjusting the concentration of the samples to 1% (w/v) sodium dodecyl sulfate (SDS, Fisher Scientific Co.), 1% (v/v) 2-mercaptoethanol (Eastman Organic Chemicals) and heating at 70 or 100 C for 3 min. When necessary, sucrose was added to 12% (w/v) to facilitate overlayering of the agarose sample gel with water as follows. The disrupted samples were mixed at 45 C with an equal volume of a molten solution of 1% agarose (Bausch and Lomb) in 0.01 M sodium phosphate, *p*H 7.2, and then layered at 37 C onto the 10% gels, overlayered with 0.1 ml of water and allowed to solidify at room temperature. Fifty μliters of a 10% sucrose solution, containing 0.005% bromophenol blue dye, was layered on top of the sample gel as an electrophoresis marker. The agarose sample gel allowed recovery of the nonmigrating material at the top of the 10% polyacrylamide gel. After electrophoresis, at 8 ma per gel for various times, an opaque "crushing plug" (10% acrylamide, 1% bisacrylamide) was polymerized on top of the agarose sample gel. The gels were fractionated top first, and assayed for radioactivity by using methods previously described (14).

Cosedimentation of RNP and rhinovirus 14. A suspension of ^{3}H-amino acid mixture-labeled SR-RSV and ^{14}C-amino acid mixture-labeled purified rhinovirus 14 was adjusted to 0.25% (v/v) NP-40 and 1% (w/v) DTT in Tris-EDTA buffer at 4 C. The detergent-treated viruses were layered immediately in the cold onto a chilled 4.8-ml 10 to 30% (w/w) linear glycerol gradient containing Tris-EDTA buffer and 0.4% (w/v) DTT. After centrifugation in a Spinco SW65 rotor at 45,000 rev/min, 4 C for 40 min, 0.125-ml fractions were collected through a needle puncturing the bottom of the tube and then directly into vials containing 0.4 ml of water for assay of radioactivity.

Precipitation and density gradient analysis of RNA. RNA was isolated from purified RSV, RNP, and soluble fraction by dissociating it from other virion components in 0.1% (w/v) SDS at room temperature,

adding 300 to 500 μg of unlabeled yeast RNA as a carrier, and precipitating with about 2 volumes of 95% (v/v) ethanol at 4 C overnight. Flocculation was aided by adjusting the solution to a final concentration of 0.1 M with ammonium acetate. The precipitates were pelleted at 4 C; washed once with cold 67% ethanol in Tris-EDTA buffer, containing 1% SDS and 0.1 M ammonium acetate; dissolved in 0.4 ml of Tris-SES buffer (0.01 M Tris-hydrochloride, *p*H 7.4, 0.05 M NaCl, 0.001 M EDTA, and 0.1% [w/v] SDS); and layered onto 10 to 30% (w/w) linear glycerol gradients containing Tris-SDS buffer. After centrifugation in a Spinco SW50 rotor at 45,000 rev/min, 20 C for 1 hr, the gradients were fractionated from the bottom directly into vials containing 0.4 ml of water and assayed for radioactivity.

Assays for radioactivity. Samples from sucrose and glycerol gradients were counted in B-10 scintillation solvent prepared as previously described (14). Channel settings on the Packard TriCarb liquid scintillation counter were adjusted for simultaneous counting of ^{3}H and ^{14}C. ^{3}H and ^{32}P doubly labeled samples were counted with optimal ^{3}H and ^{32}P channel settings. Appropriate corrections were made for background and spillover of ^{14}C or ^{32}P counts per minute into the ^{3}H channel.

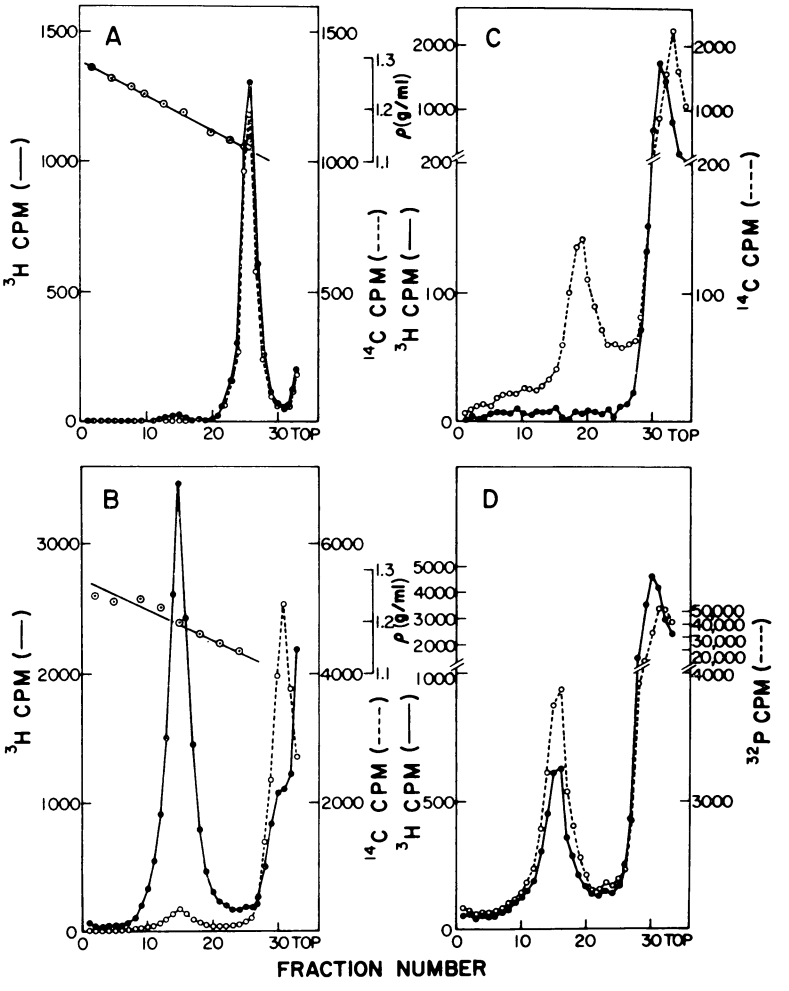

FIG. 1. *Sedimentation of NP-40-disrupted RSV in sucrose-D_2O gradients. A suspension of radioactively labeled, purified RSV was disrupted in NP-40 and layered immediately onto a 30 to 65% (w/v) sucrose-D_2O gradient as described in Materials and Methods. Untreated virus was layered onto an identical gradient. The gradients were centrifuged and assayed for radioactivity and density as described in Materials and Methods. (A) Untreated RSV, doubly labeled with ^{3}H-uridine and ^{14}C-amino acid mixture; centrifugation was for 5 hr at 45,000 rev/min. The ^{3}H counts per min-^{14}C counts per min ratio of the peak was 1.10. (B) Same as A, treated with NP-40. The ^{3}H counts per min-^{14}C counts per min ratio of the fast-sedimenting component was 9.9. (C) A mixture of ^{3}H-glucosamine-labeled RSV and ^{14}C-amino acid mixture-labeled RSV, treated with NP-40; centrifugation was for 3 hr at 45,000 rev/min. (D) RSV doubly labeled with ^{32}P- and ^{3}H-amino acid mixture, treated with NP-40; centrifugation was for 4 hr at 45,000 rev/min.*

RESULTS

Sedimentation of products of NP-40-disrupted RSV in density gradients. Purified SR-RSV, labeled by growth in the presence of different radioactive precursors, was dissociated with NP-40 and sedimented in sucrose-D_2O density gradients. Untreated virus was recovered in a single major peak near the top of the gradient (Fig. 1A). This material was converted completely by treatment with NP-40 into two components, one sedimenting rapidly, the other sedimenting slowly (Fig. 1B, 1C, and 1D). The fast-sedimenting component contained about two-thirds of the ^{3}H-uridine label (Fig. 1B, Table 1), about 10% of the amino acid mixture label (Fig. 1B, Table 1), essentially none of the glucosamine label (Fig. 1C, Table 1), and about 10% of the ^{32}P (Fig. 1D, Table 1).

Attempts to increase the recovery of RNA and protein in the fast-sedimenting component by reducing the virus harvest time from 24 hr to 6 or 12 hr after the addition of label or by adding 80 μg of unlabeled carrier virus to the labeled preparation before detergent treatment (5) were not successful. Thus our recoveries differ somewhat from the earlier report (5) that 100% of the uridine label and 30% of the leucine label were recovered in the fast-sedimenting component. The reason for this difference is unclear.

Sedimentation and isopycnic centrifugation experiments indicated that the fast-sedimenting component contained a RNP complex and was not merely a fortuitously cosedimenting mixture of RNA and protein. For example, when an RSV preparation simultaneously labeled with ^{3}H-uridine and ^{14}C-amino acid mixture was disrupted with NP-40 and centrifuged at 45,000 rev/min in a sucrose-D_2O density gradient like those in Fig. 1, the RNA and protein labels in the fast-sedimenting peak sampled at 2, 5, and 16.5 hr cosedimented (data not shown). Over this time span the fast peak sedimented from fraction number 20 (2 hr) to fraction number 6 (16.5 hr).

In another set of experiments, the doubly labeled fast-sedimenting peak was banded to equilibrium in a very dense sucrose-D_2O gradient (Fig. 2). After 24 hr of centrifugation (Fig. 2A), the fast-sedimenting peak was recovered at a density of approximately 1.34 g/ml, and the RNA and protein labels cobanded. It remained at this density even after 24 additional hours of centrifugation (Fig. 2B). A slight skewing of the protein label to the light side of the peak suggested that a small amount of protein had dissociated from this complex after 48 hr of centrifugation. A buoyant density of about 1.34 g/ml is strong evidence that the RNA and protein are complexed. The buoyant density of protein is about 1.30 g/ml, whereas that of free RNA is about 1.80 g/ml, too dense to band in the gradient.

TABLE 1. *Distribution of radioactive labels between fast- and slow-sedimenting products of Nonidet P-40-treated Rous sarcoma virus virions*

Radiolabeled precursor	Percent of total label recovered	
	Fast-sedimenting peak	Slow-sedimenting peak
^{3}H-uridine[a]	67.1 ± 0.8	33.4 ± 0.8
^{14}C-amino acid or ^{3}H-amino acid mixture[b]	8.7 ± 1.0	91.3 ± 1.0
^{3}H-glucosamine	1.0	99.0
^{32}P-orthophosphate[c]	10.9	89.1

[a] Average of four experiments on two different ^{3}H-uridine-labeled SR-RSV preparations harvested 24 hr after the addition of label ± the average deviation.

[b] Average of 10 experiments with two different ^{3}H- and two different ^{14}C-amino acid-labeled SR-RSV preparations harvested 24 hr after the addition of label. In one experiment with ^{32}P- and ^{3}H-amino acid mixture-labeled SR-RSV (see below), 11% of the ^{3}H-amino acid mixture label was recovered in the fast-sedimenting peak (Fig. 1D).

[c] Data from one experiment in which SR-RSV was simultaneously labeled with ^{32}P- and ^{3}H-amino acid mixture. Infected labeled culture fluid was harvested at two 12-hr intervals during the second day of incubation in ^{32}P-containing medium (Fig. 1D).

The sedimentation coefficient of the fast-sedimenting peak from NP-40-treated RSV was estimated to be about 130*S* by comparing its sedimentation velocity to that of rhinovirus 14 in a glycerol density gradient as follows: $r_2/r_1 \times 158S = 130S$ (Fig. 3). In this calculation r_2/r_1 is the distance traversed by the fast-sedimenting RSV peak relative to that traversed by the rhinovirus 14 peak, and 158*S* is the sedimentation coefficient of rhinovirus 14 (13). Similar results were obtained using rhinovirus 2 and poliovirus type 1 as markers. Strictly speaking, this calculation is correct only for particles with identical buoyant densities in an isokinetic gradient (16). The buoyant density of rhinovirus 14 in sucrose (1.32 g/ml, K. C. Medappa, *personal communication*) is similar to that of the RSV component. Although the glycerol gradient was not isokinetic, the error so introduced is probably appreciably less than 10% because the sedimentation positions of the two components are so similar.

Electrophoresis on polyacrylamide gels of ^{32}P-labeled RSV and its NP-40 disruption products.

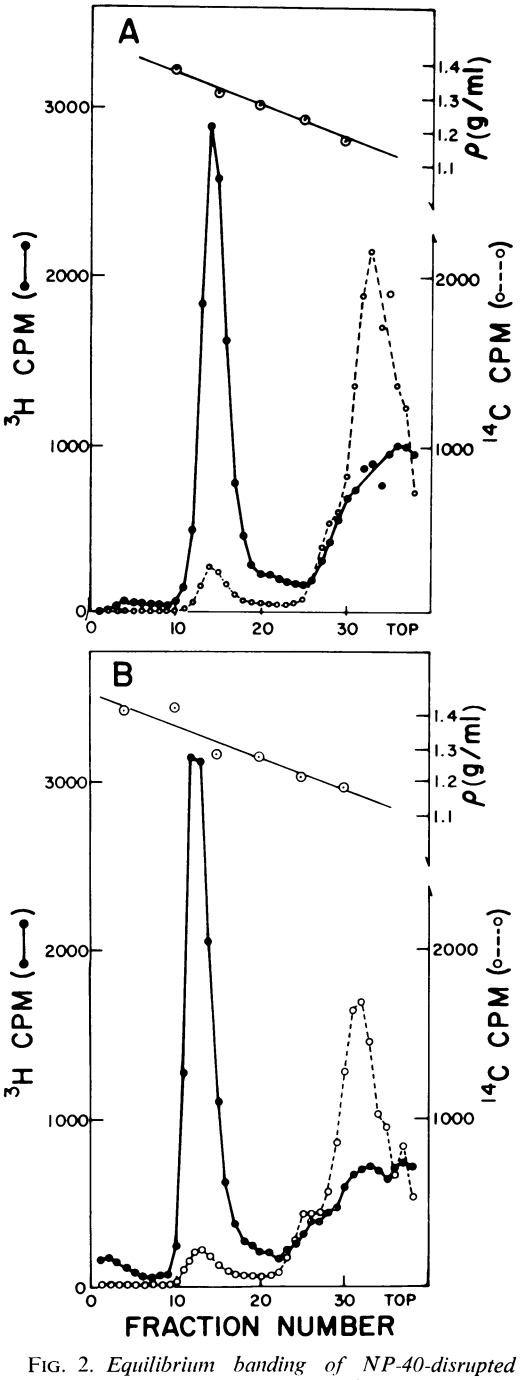

FIG. 2. *Equilibrium banding of NP-40-disrupted RSV in sucrose-D_2O gradients. Duplicate samples of RSV simultaneously labeled with ^{3}H-uridine and ^{14}C-amino acid mixture were treated with NP-40 and layered onto two chilled 30 to 90% (w/v) sucrose-D_2O gradients as described in Materials and Methods and centrifuged at 45,000 rev/min. The gradients were fractionated after 24 (A) or 48 hr (B) and assayed for radioactivity and density as described in Materials and Methods.*

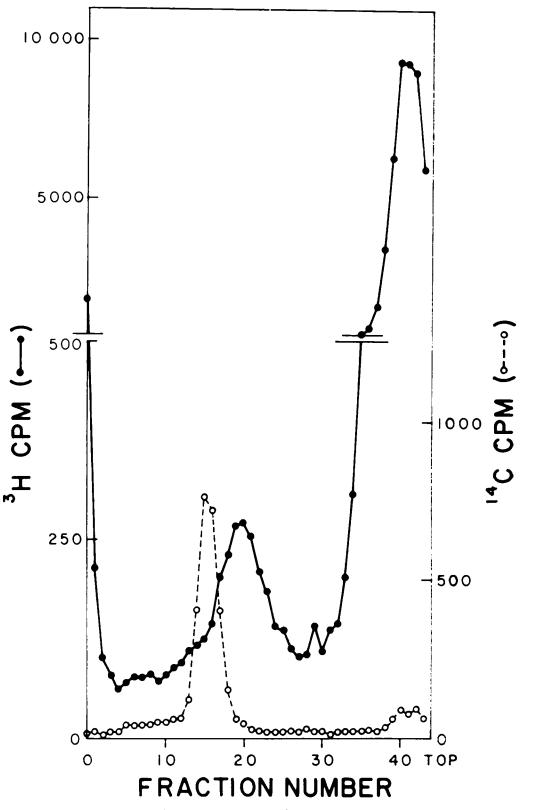

FIG. 3. *Cosedimentation of NP-40-treated RSV and rhinovirus 14. A mixture of ^{3}H-amino acid mixture-labeled RSV and ^{14}C-amino acid mixture-labeled rhinovirus 14 was treated with NP-40 and sedimented in a 10 to 30% (w/w) glycerol gradient as described in Materials and Methods. ("Fraction 0" indicates counts per minute pelleted on the bottom of the tube.)*

Dissociated ^{32}P-labeled B77 virus was resolved by electrophoresis on dodecyl sulfate-containing polyacrylamide gel into four components (Fig. 4, Table 2): high-molecular-weight (HMW; nonmigrating) RNA, 4*S* RNA, PL (phospholipid), and N (nucleotides).

In other experiments (not shown) it was demonstrated that the HMW RNA and 4*S* RNA components were alkali-labile, i.e., they were completely converted into a product, presumably 2′,3′-mononucleotides, which migrated with peak PL, when virions were heated to 100 C for 2 min at *p*H 12 before electrophoresis. The HMW RNA peak trapped at the top of the 10% gel represents the 60 to 70*S* viral RNA (probably dissociated into the 35*S* form by heating to 70 C) (8) and perhaps also a small amount of 18 and 28*S* ribosomal RNA (2). The B77 virus 4*S* RNA migrated to the same position as ^{32}P-labeled transfer RNA from *Escherichia coli*.

Peak PL contains the virion phospholipids, since 90% of the ^{32}P label extracted from virions

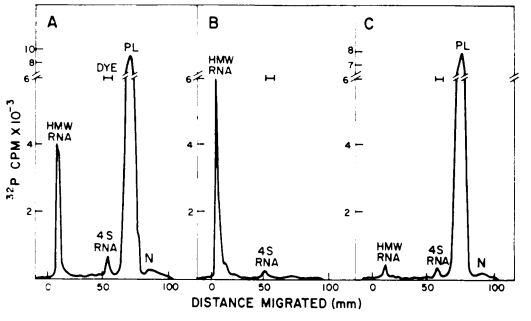

FIG. 4. *Polyacrylamide gel electrophoresis of ^{32}P-labeled B77 virus and of the fast- and slow-sedimenting components isolated from NP-40-treated virions. ^{32}P-labeled B77 virus was treated with NP-40, and the fast- and slow-sedimenting components were separated by centrifugation in a sucrose-D_2O gradient as described in Materials and Methods. Untreated virions were sedimented in a parallel gradient. The respective peak fractions were pooled, dissociated in hot 1% SDS and mercaptoethanol, electrophoresed through parallel 10-cm polyacrylamide gels, fractionated, and counted as described in Materials and Methods. HMW RNA, high-molecular-weight RNA; PL, phospholipid; N, nucleotides; dye, bromphenol blue marker dye. A, B77 virus; B, fast-sedimenting component; C, slow-sedimenting component.*

into chloroform-methanol (1:2) migrated to this position in similar gels. The remaining label resided in nonmigrating alkali-labile material and was assumed to be residual RNA transferred from the organic-aqueous interface during the extraction procedure (data not shown). As indicated above, peak PL also may contain nucleoside monophosphates which are present in purified virions in low amounts (15).

Peak N probably contains ribo- and deoxyribonucleoside triphosphates whose presence in purified RSV virions has been reported (15), since the following radiolabeled nucleotides migrated to this position: adenosine-, cytidine-, thymidine-, guanidine-, deoxyriboadenosine-, deoxyriboguanosine-, and deoxyribocytosine-triphosphate (data not shown).

Comparison of Fig. 4B and 4C (see also Table 2) shows that most of the HMW RNA as well as some 4S RNA was found in the fast-sedimenting component. Essentially all of the phospholipid was found in slow-sedimenting material. The absence of phospholipid and of glucosamine (Table 1) from the fast-sedimenting peak suggests that the virus envelope, which is known to contain these materials (18, 19), has been stripped completely from the RNP particle by NP-40.

The number of 4S RNA molecules in the virion and in the RNP can be estimated from the distribution of ^{32}P counts per minute between the HMW RNA and the 4S RNA peaks, if it is assumed that the specific activities of phosphorus in HMW RNA and 4S RNA are similar. The distribution of ^{32}P counts per minute between total RNA and phospholipid agrees very closely with the distribution of phosphorus atoms between RNA and phospholipid calculated from the chemical composition of the virus (Table 2, footnote b). Thus, under the labeling conditions used, the specific activities of phosphorus in RNA and phospholipid are similar. This result argues that the specific activities of phosphorus in HMW RNA and 4S RNA are probably also similar.

The number of 4S RNA molecules in B77 virus and in its RNP are shown in Table 3. The B77 virus RNP particle contained only 30% of the 4S RNA molecules present in the virion. Gel electrophoresis studies on ^{32}P-labeled SR-RSV gave

TABLE 2. *Distribution of label among the electrophoretic peaks of ^{32}P-labeled B77 virus and of the fast- and slow-sedimenting components produced by Nonidet P-40 treatment*

Electrophoretic peak	Percent of recovered radioactivity[a]		
	Virons[b]	Fast-sedimenting peak	Slow-sedimenting peak
HMW RNA	14.4	95	1.6
4S RNA	2.7	5.3	1.9
PL	80	<1	94
N	2.4	<1	2.2

[a] Data from Fig. 4.

[b] Rous sarcoma virus (RSV) contains 21% phospholipid and 1.9% RNA (18). Assuming each virion contains one complement of 60 to 70S RNA weighing 10^7 daltons (7), this complement represents (14.4%/[14.4% + 2.7%]) 84% of the total viral RNA; then the total weight of RNA per virion is $10^7/0.84$ or 1.2×10^7 daltons. The molecular weight of this virion would be $1.2 \times 10^7/0.019$ or 6.3×10^8, and it would contain 0.21×630 or 130×10^6 daltons of phospholipid. From the base composition of its RNA (20), the weight average molecular weight of an RSV nucleotide is calculated to be 344 daltons. Thus the RNA would represent $1.2 \times 10^7/344$ or 35,000 phosphorus atoms. Similarly, the number of phosphorus atoms in phospholipid is calculated to be $130 \times 10^6/750$ or 173,000 per virion. Therefore, the chemical data indicate that 35/(35 + 173) or about 17% of the phosphorus is in RNA and about 83% is in phospholipid. The distribution of ^{32}P counts per minute between the viral RNA and phospholipid calculated from the data in the column labeled "virions" is ([14.4% + 2.7%]/14.4% + 2.7% + 80%]) 17.5% in RNA and [80%/[14.4% + 2.7% + 80%]) 82.5% in phospholipid. This result corresponds closely to the distribution of phosphorus atoms calculated from the chemical composition as described in the above paragraph.

similar results (Table 3); the SR-RSV RNP contained 40% of the 4S RNA in the virion.

It has been reported that some 4S RNA is bound in a heat-labile complex with viral 60 to 70S RNA (8). The data in Table 3 were obtained by heating the gel samples for 3 min at 70 or 100 C before electrophoresis. Either treatment is sufficient to completely release "bound" 4S RNA (8). To determine whether any of the 4S RNA was, in fact, originally bound to a 60 to 70S RNA complex in the virus, duplicate samples of ^{32}P-labeled SR-RSV and of its RNP were dissociated in SDS and 2-mercaptoethanol to release the RNA, one set at 100 C for 3 min and the other at room temperature for 1 hr, and then subjected to electrophoresis without an agarose sample gel. The gel profile of the unheated sample of the RNP displayed only 40% as much 4S RNA as the heated one; thus 60% of the 4S RNA, corresponding to 0.60 × 30, or about 18 4S RNA molecules remained bound to the unheated HMW RNA from the RNP (data not shown). Similarly, a comparison of gels from heated and unheated SR-RSV showed that 25% of 70, or about 17 of the 4S RNA molecules, remained bound to the unheated HMW RNA from virions. Thus, the HMW RNA, whether it originated from the whole virion or its NP-40-generated RNP, was complexed with 17 to 18 molecules of 4S RNA when released by SDS at room temperature.

TABLE 3. *Calculation of the number of 4S RNA molecules per Rous sarcoma virus (RSV) virion and per ribonucleoprotein particle*

Virions and particles	Percent of ^{32}P counts/min recovered in RNA in:		No. of 4S RNA molecules per 10^7 daltons HMW RNA[a]
	HMW RNA	4S RNA	
	(1)	(2)	(3)
B77 virion[b]	84	16	70
B77 RNP[b]	95	5.3	20
SR-RSV virion[c]	84	16	70
SR-RSV RNP[c]	92	7.7	30

[a] The weight average molecular weight of an RSV nucleotide is 344 (Table 2); 10^7 daltons of HMW RNA therefore represents $10^7/344$ or 29,000 phosphorus atoms. RNA (4S) contains an average of 80 phosphorus atoms (27). Therefore, the number of 4S RNA molecules per 10^7 daltons of HMW RNA equals (percent ^{32}P counts per minute in 4S RNA/percent ^{32}P counts per minute in HMW RNA) × (29,000 phosphorus atoms in HMW RNA/80 phosphorus atoms in 4S RNA). This number represents the number of 4S RNA molecules per particle, if it is assumed that the virion and its RNP particle each contain a single complement of 60 to 70S RNA weighing 10^7 daltons.

[b] Data from Fig. 4.

[c] Data from Fig. 6.

RNA content of the ribonucleoprotein particle. The relative RNA and protein content of the RNP particle was estimated by using one preparation of SR-RSV which had been simultaneously labeled with ^{3}H-uridine and ^{14}C-amino acid mixture. Assuming that ^{3}H and ^{14}C were incorporated specifically into RNA and protein, respectively, then the composition of the RSV RNP particle can be calculated as follows. The ^{3}H counts per minute-^{14}C counts per minute ratio of the intact virion was 1.10; that of the RNP isolated from it was 9.9 (see legend to Fig. 1). Since the weight ratio of RNA-protein in intact RSV virions is 0.03 (18), then the RNA-protein ratio (R/P) of the RNP is (9.9/1.1) × 0.03 = 0.27. Moreover, if the RNP, shown above to lack phospholipid and glucosamine (Tables 1 and 2), consists only of RNA and protein, then R + P = 1.0, and, since R/P = R/(1 − R) = 0.27, one calculates that R = 0.21 (i.e., that the RNP contains about 21% RNA and 79% protein).

The nature of the RNA in the RNP and in the slow-sedimenting or soluble fraction was also studied by sedimentation in density gradients (Fig. 5). Most (79%) of the 60 to 70S RNA in the virion (Fig. 5A) was recovered in the RNP particle (Fig. 5B); however, a small amount was also recovered in the soluble fraction (Fig. 5C).

In one experiment with SR-RSV, doubly labeled with ^{32}P- and ^{3}H-amino acid mixture which was harvested 12 rather than 24 hr after the addition of label, 91% of the 60 to 70S RNA and 11% of the total virus protein were recovered in the RNP fraction (RNP isolated as shown in Fig. 1D). The R/P ratio calculated for this RNP preparation was 0.19, corresponding to an RNA content of about 16% and a protein content of 84%. The reason for this variation in the composition of the RNP is unclear.

Electrophoresis of polypeptides from RSV, RNP, and soluble fraction. The polypeptide composition of SR-RSV and its RNP was determined by polyacrylamide gel electrophoresis experiments with virions simultaneously labeled with ^{32}P- and ^{3}H-amino acid mixture (Fig. 6). The profiles of ^{32}P label contain the HMW RNA, 4S RNA, and PL peaks shown in Fig. 4. The profile of ^{3}H-amino acid mixture-labeled SR-RSV reproducibly contained 11 peaks. The RNP profile always contained five, including peaks a, c, e, f, and h. The profiles from RNP particles isolated from two other preparations of ^{3}H-amino acid mixture-labeled SR-RSV, which were harvested 24 hr after the addition of label, contained these five peaks in

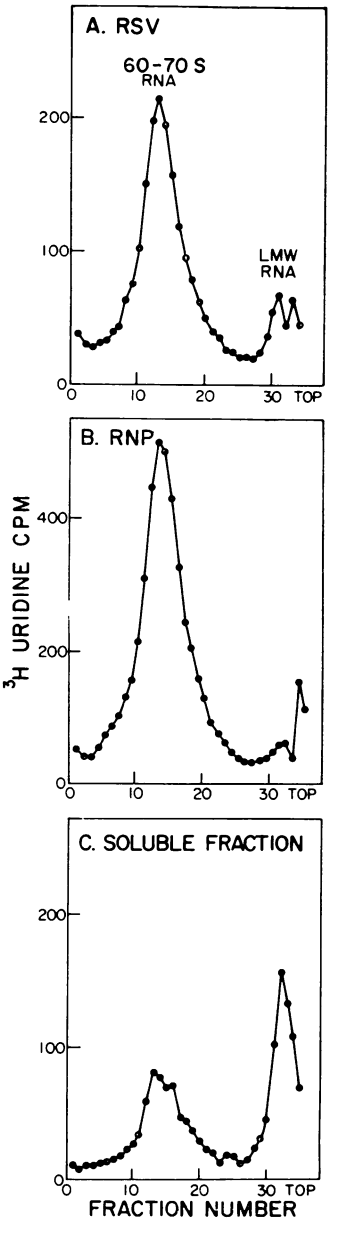

FIG. 5. *Velocity sedimentation of RNA from RSV, RNP, and soluble fraction. The RNP and soluble fraction from NP-40-treated ^{3}H-uridine-labeled RSV were separated on a sucrose-D_2O density gradient as described in Materials and Methods. An untreated control was sedimented in parallel. RNA was dissociated from virus protein and lipid in SDS, precipitated from the control and from the separated disruption products with two volumes of ethanol, and analyzed on 10 to 30% glycerol gradients as described in Materials and Methods. Sedimentation is from right to left. Rapidly sedimenting ^{3}H-uridine-labeled material is designated 60 to 70S RNA, because it could be completely converted to a slower-sedimenting form (presumably 35S RNA) by heating the gradient sample at 100 C for 3 min and quickly cooling it before layering (data not shown). LMW RNA, low-molecular-weight, or nonsedimenting, RNA.*

similar proportions. Polypeptides f and h correspond in size to two of the internal group-specific antigens of the virus (9, 10); the latter is the most abundant polypeptide in the RNP (Fig. 6).

If the RNP particle were an intact virus substructure, its polypeptide composition would be the same as it is within the virion, i.e., the number of polypeptide chains per RNP should equal the number of "RNP polypeptide chains" per virion. This was found to be true for only one polypeptide, h. A comparison between the polypeptide compositions of the virion and its RNP (Table 4) shows that the RNP contained essentially all of the h molecules originally present in the virion (2,300 h molecules per RNP particle compared to 2,500 h molecules per virion). Thus an intact RNP complex containing the viral RNA and a 14,000-dalton polypeptide was present in the RNP fraction, and this polypeptide is the one most tightly associated with the viral RNA.

The small proportion of the total h chains which is recovered in the soluble fraction (Fig. 6) probably comes from the complete dissociation of some unstable RNP particles by NP-40. This is consistent with the finding that some 60 to 70S RNA is also present in the soluble fraction (see above).

Some possible interpretations of the incomplete recovery of the other four RNP polypeptides, a, c, e, and f, will be discussed below.

DISCUSSION

We have presented evidence that the fast-sedimenting component isolated from NP-40-treated RSV by Coffin and Temin (5) is an RNP particle containing about 20% RNA, and that this particle has been completely dissociated from the viral outer envelope. This particle contains the viral 60 to 70S RNA and some 4S RNA in a complex with five virus polypeptides. It sediments at approximately 130S and has a buoyant density in sucrose of about 1.34 g/ml.

Several electron microscopy studies of RNA tumor virus structures have presented evidence that the central RNA-containing substructure of the particle is a coiled filament (4, 6, 11, 17). In a recent study of virion morphology, Luftig and Kilham reported the isolation of nucleoprotein strands with a buoyant density in CsCl of 1.34 g/ml from SDS-disrupted Rauscher leukemia virus (12). They proposed a model for Rauscher leukemia virus ultrastructure in which this nucleo-

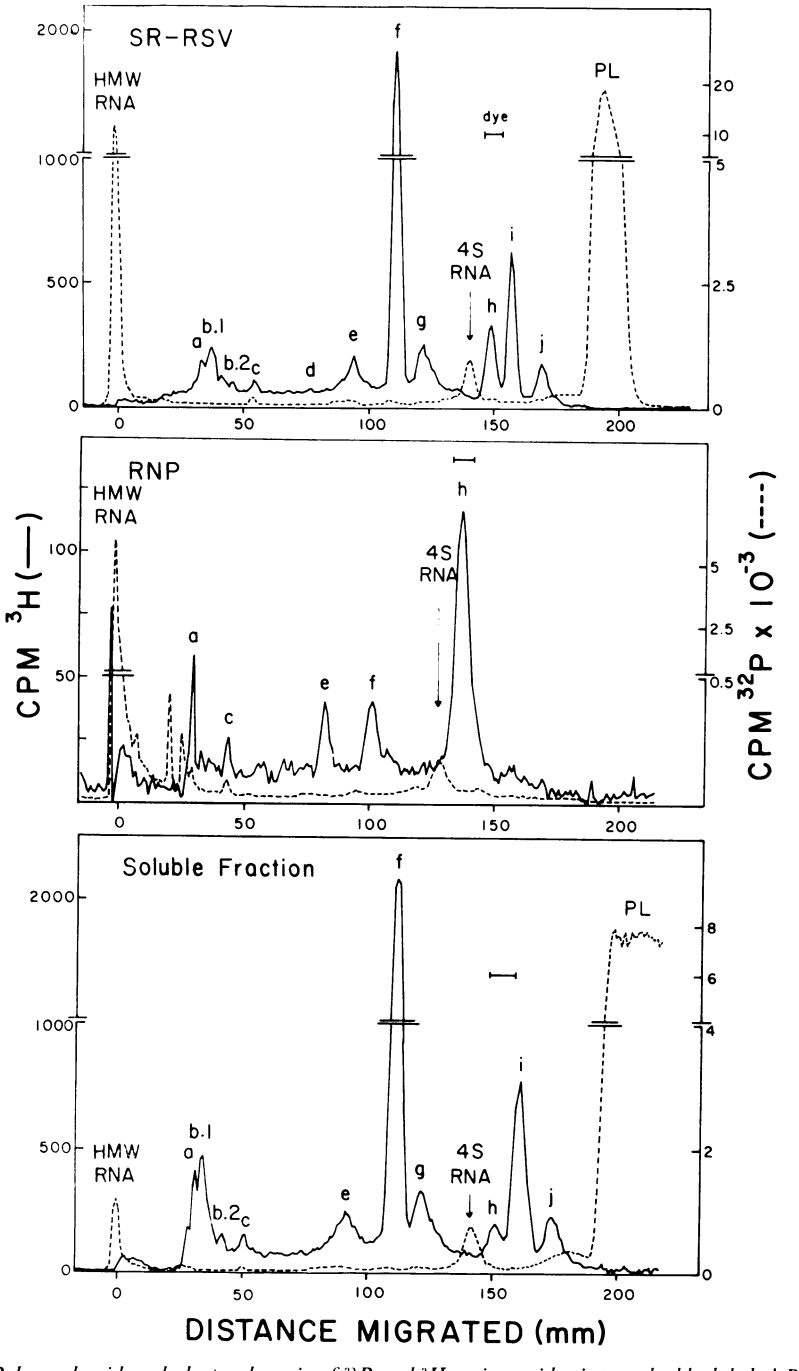

FIG. 6. *Polyacrylamide gel electrophoresis of ^{32}P and ^{3}H-amino acid mixture doubly labeled RSV, RNP, and soluble fraction. ^{32}P- and ^{3}H-amino acid mixture doubly labeled RNP and soluble fraction, isolated as shown in Fig. 1D, and a sample of the undisrupted doubly labeled RSV were dissociated in SDS and 2-mercaptoethanol and subjected to electrophoresis through 25-cm long SDS-containing 10% polyacrylamide gels, prepared and assayed as described in Materials and Methods. Polypeptide peaks in the RNP and soluble-fraction profiles were identified in other experiments by coelectrophoresis with ^{14}C-amino acid mixture-labeled RSV.*

TABLE 4. *Polypeptide composition of the SR-RSV virion and the ribonucleoprotein (RNP) released by Nonidet P-40*[a]

Peak	Apparent mol wt[b]	Virion		RNP	
		Percent total counts/min recovered	No. of chains per virion[c]	Percent total counts/min recovered	No. of chains per RNP[c]
	(1)	(2)	(3)	(4)	(5)
a	91,000	3.9	170	8.5	50
b.1	86,000	7.2	330	0.0	0
b.2	76,000	1.6	80	0.0	0
c	64,000	2.9	180	6.1	50
d	45,000	1.4	120	0.0	0
e	35,000	10.3	1,200	12.3	200
f	26,000	35.8	5,500	15.5	340
g	22,000	8.8	1,600	0.0	0
h	14,000	8.9	2,500	57.6	2,300
i	12,000	14.0	4,700	0.0	0
j	9,600	5.2	2,200	0.0	0

[a] Data from Fig. 6.

[b] The size of each polypeptide was calculated from its electrophoretic mobility in an SDS-gel relative to the mobilities of phosphorylase a (94,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carboxypeptidase A (34,600 daltons), and lysozyme (14,300 daltons) (26).

[c] Calculated by multiplying the weight of total protein in the particle by the molar ratio of each polypeptide in the particle.

The weight of protein in each particle was calculated from its RNA-protein ratio (R/P). Assuming each particle contains a single RNA genome (10^7 daltons), and, taking the 4*S* RNA content of each particle into account, this genome represents 84% of the RNA in the virion and 92% in the RNP (Table 3, column 1). Then the total amount of RNA per virion is $10^7/0.84$ or 1.2×10^7 daltons. Since R/P = 0.03 (see Results), the weight of protein, P, in the virion is $R/0.03 = 1.2 \times 10^7/0.03$ or 400×10^6 daltons. Similarly, the protein content of the RNP (R/P = 0.19) is $10^7/(0.92 \times 0.19)$ or 57×10^6 daltons. The molar ratio of each polypeptide was calculated, assuming radioactivity is proportional to mass, by dividing the percent total counts per minute recovered in each peak by its apparent molecular weight (e.g., column 2 or 4/column 1). For example, the number of h chains per virion was calculated to be $400 \times 10^6 \times (0.089/[14 \times 10^3])$ or 2,540.

protein strand, containing the viral RNA, is coiled within an inner envelope, or intermediate membrane, which in turn is surrounded by the lipoprotein outer envelope. (For purposes of this discussion, we will designate the nucleoprotein strand the "nucleoid," and call the more complex subparticle, consisting of the nucleoid enclosed in an intact intermediate membrane, the "core.") Sarkar et al. proposed a closely related general model for RNA tumor virus ultrastructure in which the filamentous nucleoid is originally packaged within the virus core in a helical configuration (22).

The RNP substructure of RSV which we studied has properties which might be predicted for the filamentous nucleoid described in these models. It contains the RNA genome in an intact complex with a polypeptide of 14,000 molecular weight, which corresponds in size to an internal (group-specific) virus antigen (9, 10). It also has a buoyant density in sucrose of 1.34 g/ml, and enzyme activities such as an endogenous ribonuclease-sensitive deoxyribonucleic acid (DNA) polymerase activity and a DNA-directed DNA polymerase activity, which may be involved in the process of virus infection (5).

The polypeptide composition data presented in this study leave unresolved the question of whether (i) the nucleoid consists solely of the complex between the viral RNA and the 14,000 molecular-weight polypeptide or (ii) the nucleoid contains in addition one or more of the other four polypeptides found in the RNP, a, c, e and f. In the first case, the presence of the four incompletely recovered polypeptides could be ascribed to adherent fragments of an incompletely removed superstructure encapsulating the nucleoid. In the second case, the incomplete recovery of one or more nucleoid components may reflect their easier dissociation by NP-40. In any case, it is probable that the 14,000-dalton polypeptide is a major, if not the only, structural protein of the nucleoid.

Other groups have studied the polypeptide composition of RNP substructures isolated from NP-40-treated avian tumor virus with results very similar to ours. Robinson and Robinson reported the major polypeptide in a fast-sedimenting component from NP-40-treated Bryan strain RSV-(RAV-1) to be a 16,500 molecular-weight species (21). Fleissner, using gel filtration in 6 M guanidine hydrochloride, observed a single 12,000 molecular-weight protein in a subparticle isolated from NP-40-treated MC29 virus (*personal communication*).

Smith and Joklik reported the isolation of a similar RNA-containing subviral particle which lacked glucosamine, and contained seven of the 11 virus polypeptides, from the Prague strain of RSV after treatment with Triton X-100 detergent (Abstr. Annu. Meeting Amer. Soc. Microbiol., 1972, p. 224).

Bolognesi et al. isolated virus cores from a large amount of avian myeloblastosis virus (2×10^{13} particles) by disruption in NP-40 followed by extraction with ether (3). Polyacrylamide gel electrophoresis of cores isolated by this procedure revealed two main polypeptides with molecular weights of 28,000 and 30,000. If the 14,000 molecular-weight polypeptide is a nucleoid protein as we have suggested and if Bolognesi et al. have isolated an intact core, their gel profiles should show the 14,000 molecular-weight polypeptide. The reason for this inconsistency will have to be clarified by further investigation.

Erikson and Erikson reported the presence of 4*S* RNA molecules in a heat-labile complex with 60 to 70*S* RNA isolated from avian myeloblastosis virus by phenol extraction (8). Their experiments with ^{32}P-labeled RNA indicated that about five molecules of 4*S* RNA were complexed per 35*S* RNA molecule. This result suggests that 15 to 20 molecules of 4*S* RNA may be included in the 60 to 70*S* RNA complex, assuming that the 60 to 70*S* RNA contains three or four 35*S* RNA molecules (7). The fact that we have obtained similar results with RNA from SDS-dissociated SR-RSV and its RNP confirms this report and suggests that this complex is a real component of the virion, and not an artifact induced by phenol extraction.

The techniques we have described in this study of the RSV RNP substructure, or "nucleoid," may now be fruitfully applied in the study of cores to illuminate the structural organization of the RNA tumor virus.

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