*ISOLATION OF NONINFECTIOUS PARTICLES CONTAINING ROUS SARCOMA VIRUS RNA FROM THE MEDIUM OF ROUS SARCOMA VIRUS-TRANSFORMED NONPRODUCER CELLS**

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Stocks of the Bryan high-titer strain of Rous sarcoma virus (RSV) have been shown to contain avian leukosis viruses as well as RSV.[1] Chick embryo fibroblasts infected with the leukosis viruses associated with the Bryan stocks, Rous associated virus-1 (RAV-1) or Rous associated virus-2 (RAV-2), do not transform but do produce virus, whereas cells infected with RSV transform but do not produce infectious virus.[2] When RSV-infected transformed cells are superinfected with RAV-1 or RAV-2, the transformed cells not only produce the superinfecting virus but also produce infectious RSV which has the growth characteristics,[3] antigenic specificity,[4] and host range of the superinfecting virus.[5] The RSV-transformed cells which do not produce virus have been named nonproducer (NP) cells, and the avian leukosis viruses associated with the Bryan stock, helper viruses. Viral coat antigen cannot be detected in NP cells,[2, 4] and it has been hypothesized that the absence of coat antigen in RSV-infected cells prevents synthesis of infectious RSV and that the role of the helper virus in the production of infectious RSV is to provide coat antigen.[2] The finding that NP cells do not produce infectious virus raised the possibility that the carcinogenic action of RSV might be related to the apparent absence of late functions in virus replication in infected cells.[2]

Recently, Dougherty and DiStefano[6] have observed particles with the appearance of avian leukosis viruses in electron micrographs of RSV-transformed NP cells and have suggested that NP cells may be producing non infectious virus.

The experiments presented here approach the question of virus production by the NP cell with the aid of radioactive tracers[7] and demonstrate that the NP cell is producing noninfectious particles with the physical characteristics of the avian leukosis viruses. The nucleic acid of the particles is characterized and found indistinguishable in sedimentation velocity and base composition from the RNA of RSV + RAV.

Materials and Methods.—Isolation and care of NP cells: Chick embryo cells from 10-day-old embryos of strain 13 or 813 white leghorn chickens which were susceptible to infections by both group 1 and group 2 avian leukosis viruses were used throughout the course of this work. The methods used for culturing chick embryo cells[9] and assaying for focus-forming units of RSV[9] and interfering units of RAV[1] have been described. Clones of NP cells were isolated according to the method of Trager and Rubin[8] from secondary chick embryo fibroblasts infected with the Bryan high-titer strain of RSV. The isolated clones were grown on X-irradiated chick embryo fibroblast feeder layers (4×10^5 cells/60-mm plate) in a rich medium: 82 per cent 199A, 10 per cent tryptose phosphate broth, 4 per cent calf serum, 1 per cent chicken serum, 2 per cent 2.8 per cent $NaHCO_3$, 1 per cent beef embryo extract, which had been incubated at 57°C for 45 minutes to inactivate any contaminating leukosis viruses. Culture medium was changed daily and the cultures

were maintained at pH 7.2–7.6 by repeated additions of 2.8 per cent $NaHCO_3$. Clones were passed every four to six days when the original seeding of about 2×10^5 cells per 60-mm plate had grown to over 10^6 cells. At passage, control cultures from each clone were superinfected with 10^6 interfering units of RAV-1. Cultures were used for experiments three or more days after passage when there were 10^6 or more cells per plate and when the production of focus-forming units by the RAV-challenged cultures had reached a maximum.

Radioactive precursors and labeling of virus: H^3 uridine (24 c/mmole) was purchased from New England Nuclear and used at concentrations of 40–100 μc/ml in growth medium which contained 199A minus uridine and cytosine. Carrier-free $P^{32}O_4$, purchased from New England Nuclear, was used at concentrations from 0.2 to 1.0 mc/ml in growth medium which contained 95 per cent PO_4 free medium (prepared with 199A minus phosphate and tryptose broth) and 5 per cent regular growth medium.

The methods used in virus purification and in isolation and characterization of viral RNA are those described by Robinson *et al.* for RSV + RAV.[7] Any modifications of these methods are presented in the text and figure legends. The buffer used for dilution of intact virus and preparation of w/v sucrose solutions was 0.1 *M* NaCl, 0.05 per cent β mercaptoethanol, 0.001 *M* ethylenediaminetetraacetic acid (EDTA), 0.01 *M* tris, pH 7.4. RNA extractions were done in the same buffer minus β mercaptoethanol and plus 1 per cent sodium dodecyl sulfate (SDS).

Results.—In the experiment shown in Figure 1, cultures which contained approxi-

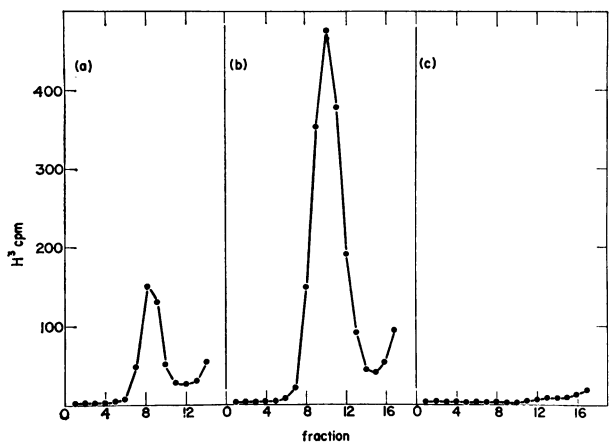

FIG. 1.—Incorporation of H^3 uridine into particles isolated from the medium of an NP culture, its RAV-challenged sister culture, and a culture of normal cells. Approximately 10^6 NP cells (*a*), NP cells which had been challenged one week prior with 10^6 interfering units of RAV-1 (*b*), and normal chick embryo fibroblasts (*c*) were incubated in 5 ml medium containing 100 μc H^3 uridine. At 18 hr the medium was harvested and centrifuged at 15,000 rpm for 10 min in the Sorvall RC-2 to remove cell debris, following which 4 ml was layered over 7 ml of 10% sucrose which had in turn been layered over a 5-ml linear gradient from 15 to 60% sucrose. One ml was saved for virus assay. Centrifugation was for 4 hr at 25,000 rpm and 6°C in the SW 25.3 rotor of the Spinco preparative ultracentrifuge. The gradients were collected dropwise from the bottom of the tubes; 0.05 ml of each 0.2-ml sample was mixed with 0.1 ml H_2O and 5 ml Bray's solution for liquid scintillation counting.[7] No focus-forming units were detected in 1 ml of medium from the nonproducer cells or the control cells. The medium from the RAV-challenged NP culture contained 2×10^6 focus-forming units/ml.

mately 10^6 NP cells (*a*), NP cells which had been superinfected seven days prior with 10^6 infectious units of RAV (*b*), and normal chick embryo fibroblasts (*c*) were incubated for 18 hours in the presence of H^3 uridine. The medium from these cultures was examined for the presence of radioactivity in particles with the sedimentation characteristics described by Robinson and co-workers for a number of lipid-containing RNA tumor viruses.[7, 10–12] The medium harvested from the culture of NP cells (Fig. 1*a*) contained particles which sedimented like the large lipid-containing RNA viruses. The amount of H^3 uridine present in the particles was about one fourth that found in RSV + RAV in the medium from the RAV-challenged sister culture of the NP culture (Fig. 1*b*). No H^3 uridine in particles was detected in medium from the control culture (Fig. 1*c*).

The incorporation of radioactive precursors of RNA into rapidly sedimenting particles and the release of these particles into the culture medium appears to be a property of all NP cells isolated by us from the Bryan high-titer strain of RSV. Throughout the course of this work 14 NP clones have been examined and found to produce particles.

The data in Table 1 present the titers of RSV and of RAV, and the amount of H^3 uridine incorporated into RSV + RAV or into NP particle contained in the medium of two clones of NP cells and their RAV-challenged sister cultures. No infectious RSV or RAV was detected in the medium from the NP clones whereas over 10^7 focus-forming units of RSV and interfering units of RAV were present in the medium from the NP clones superinfected with RAV. In contrast to this, more than 10^7-fold difference in the titers of infectious RSV and RAV found in the medium of the NP clones and their RAV-challenged sister cultures, there was less than a twofold difference in the amount of H^3 uridine incorporated by the NP clones and their RAV-challenged sister cultures into NP particle or into RSV + RAV. From the data in Table 1 it can also be seen that the assays for focus-forming units and interfering units in the medium from the RAV-challenged cultures are from 1,000 to 10,000 times more sensitive than the assay for the incorporation of H^3 uridine into RSV + RAV. In fact, the possibility that the NP particle represented low levels of infectious RSV or RAV which were being detected by the incorporation of H^3 uridine into particles but not by the assays for focus-forming units or inter-

TABLE 1

PRODUCTION OF NP PARTICLE AND RSV + RAV BY TWO NP CLONES AND THEIR RAV-CHALLENGED SISTER CULTURES

	Clone A*		Clone B*	
	NP cells	NP cells superinfected with RAV-1	NP cells	NP cells superinfected with RAV-1
Cell number	7×10^5	9×10^5	7×10^5	7×10^5
RSV, focus-forming units†	0	3.1×10^7	0	2.7×10^7
RAV-1, interfering units†	0	7.5×10^7	0	1.3×10^8
RAV-2, interfering units†	0	0	0	0
H^3, cpm in NP particle or in RSV + RAV‡	4.0×10^3	7.1×10^3	3.1×10^3	4.6×10^3
H^3, cpm in cell RNA‡	2.3×10^6	2.5×10^6	2.3×10^6	2.2×10^6

* Cultures were transferred and superinfected with RAV-1 4 days before the experiment.

† Focus-forming units and interfering units present in 5 ml medium after 18 hr of growth. The titers of RAV-1 and RAV-2 were determined in an end-point assay as the number of units which when grown with chick embryo fibroblasts for three passages interfered with superinfection of the fibroblasts by either RSV with RAV-1 coat antigen or RSV with RAV-2 coat antigen.

‡ Cultures were incubated with 200 μc H^3 uridine in 5 ml of growth medium for 18 hr. Under these conditions incorporation of H^3 uridine is linear for several hours, after which it falls off due to medium exhaustion. NP particle and RSV + RAV were isolated as described in Fig. 1. Cells were solubilized in a buffer containing 2% SDS, 0.05% β mercaptoethanol, 0.1 *M* NaCl, 0.005 *M* EDTA, and 0.01 *M* tris, pH 7.4. Aliquots of the virus and cell suspension were analyzed for acid-precipitable, alkali-labile H^3 cpm.

fering units is ruled out by these data, which show that the incorporation of H^3 uridine into particles is a much less sensitive assay for RSV or RAV than the determination of focus-forming units or interfering units.

The possibility that the NP particle was a noncytopathic but infectious virus which was growing in the NP cells was tested by incubating medium from NP cells or their RAV-challenged sister cultures with normal chick embryo fibroblasts to allow any viruses present in the media to infect the fibroblasts. These fibroblasts were then grown for five days and assayed for the presence of replicating virus by determining their ability to incorporate H^3 uridine into particles. As can be seen in Figure 2, no evidence for the replication of NP particle could be found in the medium of the fibroblasts initially incubated with medium from the NP culture whereas the replication of RSV + RAV was easily detected in the fibroblasts infected with the RSV + RAV present in the medium from the RAV-challenged NP culture. In brief, it would appear that the RSV-transformed NP cell is producing RNA containing particles (Fig. 1) which are not infectious RSV or RAV (Table 1) or a noncytopathic virus growing in the NP clones (Fig. 2).

Attention was next turned to a detailed comparison of the physical properties of the particles produced by the NP cells with those of RSV + RAV and to the isolation and characterization of the RNA in the NP particle.

Physical characteristics of the NP particle: Figure 3 presents data from an experiment in which NP cultures were labeled with H^3 uridine and their RAV-challenged sister cultures with $P^{32}O_4$. The media containing H^3-labeled NP particle and P^{32}-labeled RSV + RAV were mixed. The RSV + RAV and NP particle were then isolated and sedimented in the appropriate sucrose gradients to compare their buoyant densities and sedimentation velocities. The equilibrium sedimentation data in Figure 3 indicate that the NP particle, detected by the presence of H^3 RNA, has a slightly lower buoyant density (1.170 gm/ml) than that (1.175 gm/ml) of the physical particles of RSV + RAV, detected by P^{32} RNA, which in turn have a slightly lower buoyant density than that (1.180 gm/ml) of the infectious particles of RSV + RAV, detected as focus-forming units and interfering units. In each of four such experiments radioactivity in the NP particle was found at a lower density in the gradient than radioactivity in RSV + RAV, which in turn

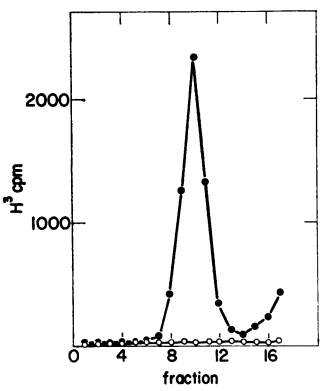

FIG. 2.—Incorporation of H^3 uridine into particles by chick fibroblasts incubated with NP particle or RSV + RAV; 0.5×10^6 secondary chick embryo fibroblasts per 60-mm plate were seeded in 5 ml medium in which NP cells or their RAV-challenged sister cultures had been grown for 24 hr. The medium from the NP cells contained no detectable focus-forming units/ml whereas the medium from the RAV-challenged culture contained 2×10^6 focus-forming units/ml. Three hours after seeding, the cultures were changed into fresh growth medium, after which they were cultured 5 days with daily medium changes. On day 5, cultures which had been incubated with 100 μc H^3 uridine for the past 18 hr were assayed for the incorporation of H^3 uridine into particles and the production of focus-forming units as described in Fig. 1. No focus-forming units were detcted in 1 ml of medium from the cultures initially incubated in NP medium while 5×10^6 focus-forming units/ml were present in the medium of the fibroblasts exposed to RSV + RAV. Open circles represent cpm H^3 in particle present in the medium of fibroblasts initially incubated with NP medium, and closed circles, cpm H^3 in particle in the medium of fibroblasts incubated in medium from the RAV-challenged NP culture.

was found at a lower density than focus-forming units.[13] The actual densities have ranged from 1.160 to 1.175 gm/ml for radioactivity in the NP particle, from 1.165 to 1.180 gm/ml for radioactivity in RSV + RAV, and from 1.173 to 1.185 gm/ml for focus-forming units. The differences in buoyant density from experiment to experiment appear to reflect the age of the virus-producing culture: virus from cultures carried more than four weeks have higher densities than virus from cultures carried two to three weeks. Experiments in which the sedimentation rate of H^3 uridine in the NP particle was compared with that of P^{32} in RSV + RAV demonstrated that the NP particle sediments slightly less rapidly than RSV + RAV. Thus, it would appear that the size and shape as well as the buoyant density of the NP particle are similar to those of RSV + RAV.

RNA from the NP particle: In the experiment shown in Figure 4 the sedimentation velocity of H^3-uridine-labeled RNA from the NP particle and P^{32}-labeled RNA from RSV + RAV have been compared by zone sedimentation in a sucrose gradient. In this experiment NP cells and their RAV-challenged sister cultures were grown in media containing H^3 uridine and $P^{32}O_4$, respectively. After eight hours of growth, the media were mixed and the NP particle and RSV + RAV were rapidly concentrated and purified from the growth media by sedimentation in the appropriate sucrose solutions. The RNA in the concentrated purified NP particle and RSV + RAV was immediately phenol-extracted in the presence of added cell RNA,[7] concentrated by ethanol precipitation,[7] and sedimented in a gradient. In Figure 4 the

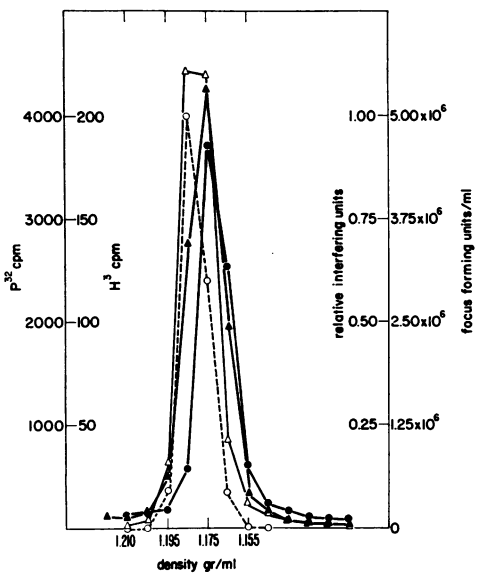

FIG. 3.—Equilibrium density gradient centrifugation of H^3-labeled NP particle and P^{32}-labeled RSV + RAV. An NP culture was incubated with 200 μc H^3 uridine, and its RAV-challenged sister culture with 2 mc $P^{32}O_4$ for 9 hr. The growth media, minus 1-ml aliquots for virus assay, were mixed, centrifuged for 10 min at 15,000 rpm in the Sorvall RC-2, layered over 0.5 ml of 60% sucrose, and centrifuged for 20 min at 48,000 rpm and 6°C in the SW 50 rotor of the Spinco ultracentrifuge. The zone between the 60% sucrose and medium, containing all the NP particle and RSV + RAV, was collected, diluted, layered over 2 ml of 20% sucrose which overlay 0.5 ml of 60% sucrose, and centrifuged for 30 min at 48,000 rpm. Three-drop fractions were collected from the bottom of the tube, and the fraction which contained the majority of the cpm of H^3 and P^{32} was diluted 1 to 4, layered over a 4.5-ml linear gradient from 15 to 60% sucrose, and centrifuged for 12.5 hr at 42,000 rpm, 6°C. Aliquots of the fractions from this gradient were diluted in growth medium for determination of focus forming units (Δ-Δ-Δ) and relative interfering units (O–O–O). Interference to superinfection by RSV with RAV-1 coat antigen was determined after one passage of the RAV-infected chick embryo fibroblast and is plotted relative to an interference of 1 arbitrarily assigned to the fraction containing the largest number of interfering units.[1] A second aliquot of each fraction was diluted to 1 ml and extracted with an equal volume of buffer saturated phenol[7] before acid precipitation. The acid-precipitable cpm H^3 in RNA in the NP particle (●–●–●) and P^{32} in RNA in RSV + RAV (▲-▲-▲) were determined in a Packard liquid scintillation counter under conditions where 0.6% of the cpm registered as P^{32} also registered in the H^3 channel. No focus-forming units were detected in 1 ml of medium from the NP culture, while 5×10^6 focus-forming units/ml were present in the medium from the RAV-challenged culture. Densities were determined by weighing 100 λ aliquots of the gradient fractions.

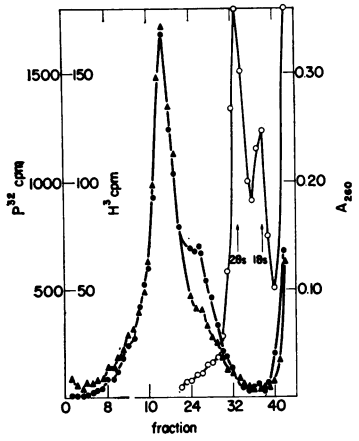

FIG. 4.—Velocity sedimentation of H^3-labeled RNA from the NP particle and P^{32}-labeled RNA from RSV + RAV. H^3-uridine-labeled NP particle and P^{32}-labeled RSV + RAV were concentrated and purified as described in the legend to Fig. 3. The procedures used during the phenol extraction of RNA from the concentrated, purified particles have been described.[7] The mixture of H^3-uridine-labeled NP RNA (●-●-●) and P^{32}-labeled RSV + RAV RNA (▲-▲-▲) was fractionated by zone sedimentation in a 17-ml linear gradient from 10 to 30% sucrose prepared in buffer containing 0.1 *M* NaCl, 0.001 *M* EDTA, 0.01 *M* tris, 0.2% SDS, pH 7.4. Centrifugation was for 6 hr at 25,000 rpm, 25°C, in the SW 25.3 rotor of the Spinco ultracentrifuge. One ml of the original NP medium contained no detectable focus-forming units while 6×10^6 focus-forming units/ml were present in the medium from the RAV-challenged NP culture. Open circles represent A_{260} of the 28 and 18*S* ribosomal RNA used as carrier.

rate of sedimentation of RNA from the NP particle is indistinguishable from that of the 71*S* RNA of RSV + RAV.[7] When similar experiments were done using virus from cultures which had been labeled more than 18 hours, ammonium sulfate to concentrate virus,[7, 10] or trypsin to release cell-associated virus, the RNA from the NP particle contained several heterogeneous components sedimenting between 30 and 71*S*[14] while the RNA from RSV + RAV sedimented at 71*S*. The RNA in the NP particle appears to be more susceptible to degradation than the RNA in RSV + RAV. It is also of interest that when RSV + RAV is purified under the conditions necessary to obtain large-molecular-weight RNA from the NP particle, (1) the RNA sediments in a narrower band than has been reported previously[7] and (2) little or no 4*S* RNA (thought to be a breakdown product of the 71*S* RNA[7]) is present.

The base composition of the 71*S* RNA from the NP particle is compared with RSV + RAV RNA in Table 2 where it can be seen that the base compositions of the RNA from the NP particle and RSV + RAV are indistinguishable. The 71*S* RNA from the NP particle is also like RSV + RAV RNA in that it is digested to acid-soluble fragments when incubated with 25 γ/ml pancreatic ribonuclease and 2 γ/ml T_1 ribonuclease at 37° for 60 minutes in 0.1 *M* NaCl.

Discussion.—The similarities in sedimentation rate and buoyant density of the NP particle and RSV + RAV and the isolation of RNA from the NP particle which is indistinguishable in sedimentation velocity, base composition, and sensitivity to ribonuclease from the RNA of RSV + RAV suggest that the NP particle may be

TABLE 2

BASE COMPOSITION OF 71*S* RNA FROM RSV + RAV AND FROM THE NP PARTICLE

	RSV + RAV RNA	NP particle RNA
C	24.2 ± 0.2	24.2 ± 0.3
A	25.1 ± 0.3	24.8 ± 0.4
G	28.3 ± 0.5	29.2 ± 0.5
U	22.4 ± 0.4	21.7 ± 0.8

Cultures were labeled with $P^{32}O_4$ for 48 hr. The RNA from the NP particle or RSV + RAV isolated from the 30–48-hr growth medium was extracted and fractionated on sucrose gradients as described in Fig. 4. The amount of P^{32} in each base was determined after mild alkaline hydrolysis of the RNA and electrophoretic separation of the nucleotides.[7] The base composition of RSV + RAV RNA was identical to that previously published.[7] Data for the NP particle represents two determinations on two independent preparations. The data is presented as the average percentage of the total cpm P^{32} present in each nucleotide plus or minus a standard error.

noninfectious RSV which is produced by RSV-infected NP cells in the absence of helper virus. Experiments to see if the genetic information of the NP particle is the same as that of the Bryan strain of RSV are now in progress.

The observations that the RNA from the NP particle, presumably RSV RNA, has the same sedimentation rate and base composition as the RNA previously characterized from a 1:10 mixture of RSV + RAV[7] and from avian myeloblastosis virus[10] indicate that RSV may have arisen from mutation(s) of an avian leukosis virus. A close genetic relationship between sarcoma viruses and leukosis viruses is also suggested in the numerous reports of the appearance of sarcomas in chickens inoculated with leukosis viruses[15, 16] and in the recent report of the apparently spontaneous appearance of a virus which causes sarcomas in mice in stocks of the Moloney mouse leukemia virus.[17]

The amount of H^3 uridine incorporated into particles by NP clones has ranged from 20 to 70 per cent (Fig. 1 and Table 1) that incorporated into RSV + RAV by their RAV-challenged sister cultures. If it is assumed that the RSV and the RAV produced by the same cell have similar ratios of physical particles to infectious particles and have labeled RNA's of the same specific activity, and that the assays for focus-forming units and interfering units have comparable efficiencies, then the titers of focus-forming units and interfering units present in the medium of a RAV-challenged NP culture should reflect the relative proportion of H^3 uridine present in RSV and in RAV. Referring to Table 1, clone A, in accordance with the infectivity assays where 30 per cent of the total infectious particles is RSV and 70 per cent RAV, let 30 per cent of the total radioactivity present in RSV + RAV (7100 cpm) be in RSV (2130) and 70 per cent in RAV (4970). Then, the amount of H^3 uridine incorporated into RSV by the RAV-challenged NP culture is comparable to that incorporated into particles by the NP culture (4000 cpm). If this calculation is valid and the NP particle is in fact noninfectious RSV, the data in Table 1 would indicate that the over-all production of RSV by NP cells is similar whether or not the cells are also infected with RAV. Thus, the role of helper virus in the synthesis of infectious RSV by NP cells would be to provide coat antigen for the RSV which is already being synthesized in a noninfectious form by the NP cells.

The demonstration that NP cells are producing noninfectious particles further defines the nature of the defectiveness of the Bryan strain of RSV. Indeed, the defectiveness of RSV does not seem to lie in the inability of the nonproducer cell to produce RNA containing particles with the physical and chemical characteristics of avian tumor viruses but rather in the inability of the particles which the NP cells produce, presumably because of the absence of coat antigen, to infect other cells. The discovery of the NP cell raised the possibility that the apparent failure of RSV to perform late functions in virus replication might be related to the transformation of infected cells by RSV.[2] The experiments presented here, which indicate that the over-all production of NP particle by RSV-transformed NP cells is comparable to the production of infectious RSV by their RAV-challenged sister cultures, suggest that many viral functions proceed normally in the absence of detectable coat antigen in NP cells.

Finally, the demonstration of actively replicating RSV RNA in NP cells makes it unnecessary to postulate an RSV provirus[18, 19] to ensure stable inheritance of RSV genetic information in NP cells.

Summary.—Chick embryo cells infected and transformed by the Bryan high-titer

strain of RSV produce noninfectious particles with physical and chemical characteristics similar to those of RSV + RAV. The single-stranded RNA contained in the particles is indistinguishable in sedimentation velocity and base composition from RSV + RAV RNA.

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† National Science Foundation postdoctoral fellow.

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[13] Physical particles of RSV + RAV, detected by P^{32}, have been previously reported to have the same buoyant density as focus-forming units.[7] The earlier studies differ from this one in that (1) NH_4SO_4 was used during virus purification, (2) the sucrose solutions were prepared in 0.01 *M* tris, 0.001 *M* EDTA, pH 7.3, (3) the virus was isolated from the culture medium of cells which had been simultaneously infected with RSV + RAV. In both studies it would appear that P^{32} was present in RSV + RAV and not in cellular material since more than 90% of the P^{32}-labeled RNA was 71*S* viral RNA (W. S. Robinson, personal communication and personal observation).

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