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Properties and Origins of Infectious Rhinovirus Type 14 Particles of Different Buoyant Densities

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Isopycnic centrifugation of rhinovirus type 14 (RV14), purified from infected HeLa or KB cell cultures, into CsCl gradients resolved two bands of infectious virus particles with buoyant density values of 1.409 ± 0.007 (H virus) and 1.386 ± 0.004 (L virus) g/ml. Only H virus was detected by incorporation of radiolabeled uridine into viral RNA, and H virus accounted for the majority of infectivity in gradients. H and L virus could not be differentiated by plaque morphology, extent of neutralization by RV14-specific antiserum, or particle size. Electron microscope studies showed that most L-virus particles were associated with an amorphous material. Treatment of L virus with proteolytic enzymes or rebanding L virus in CsCl gradients resulted in recovery of the majority of infectivity as H virus. Virus purified from cell-free fluids from infected HeLa or KB cell cultures banded only as H virus. HeLa cell cultures challenged with purified H virus and harvested at 3 h postinoculation for virus purification yielded only infectious H virus. Both H and L viruses were detected in cell cultures that had been challenged with purified H virus and harvested at 12 h postinoculation. The data suggest that H virus represents progeny virus, whereas L virus represents sequestered infectious virus particles which become associated with an amorphous material and do not enter into viral replicative processes.

Isopycnic centrifugation of picornaviruses into cesium chloride solutions and recovery of each virus at a specific buoyant density has provided a useful method of purifying virus particles for physicochemical studies. Buoyant density value is also one of several properties used in classifying viruses within this large group into subgroups (21, 25). Rhinoviruses can be distinguished from other picornaviruses on the basis of lability of infectivity at pH 5 and below, high adenylic acid content in the genome, and buoyant density of virions in cesium chloride (27). Buoyant density values for the human rhinoviruses (27) generally fall into two groups, with mean values of approximately 1.385 and 1.405 g/ml. There are many factors which affect buoyant density values of viruses in CsCl solutions and these factors have undoubtedly contributed to the ambiguity in buoyant density values reported for several human rhinoviruses (21, 25, 27). There is agreement among different laboratories on buoyant density values for at least two rhinoviruses. Rhinovirus serotype 1A has a buoyant density value in CsCl which places it in the low density group (10, 20), whereas rhinovirus type 2 has a buoyant density in CsCl which places it in the high density group (2, 3, 6, 13).

The buoyant density of rhinovirus type 14 (RV14) has been reported as 1.385 to 1.386 g/ml (13, 15) and as 1.40 to 1.408 g/ml (8, 30). The reasons for this large difference are unclear and one reason may be the reversible binding of cesium ions to RV14 virions (14; K. C. Medappa and R. R. Rueckert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, V43, p. 207). Studies in this laboratory do not resolve this disagreement, however, it has been found that infectious RV14, purified from infected HeLa or KB cell cultures, bands at two positions in CsCl gradients with average buoyant density values of 1.409 and 1.386 g/ml. The properties and origins of virus particles within these two bands were examined. Data is presented which support the conclusions that the more dense virus particles are progeny virus and the less dense virus particles represent virions which become sequestered by the host cells, reversibly altered by

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attachment of an amorphous material, and do not participate in replicative processes during one complete virus growth cycle.

MATERIALS AND METHODS

Cells and virus. Methods for the culture of HeLa (rhino-HeLa) and KB cells were previously described as well as methods for preparing RV14 stocks and plaque assay of RV14 (26). Procedures for propagation and plaque assay of reovirus type 3 in L cells were previously described (23).

Preparation and purification of radioactive-labeled RV14. Propagation of RV14 in cells in the presence of either [2-^{14}C]uridine (0.1 μCi/ml) or [5-^{3}H]uridine (1.0 μCi/ml) was carried out as previously described (8). Briefly, cells were challenged with 20 to 50 PFU per cell of RV14. After a 2-h period for adsorption of virus, the inoculum was removed. The cells were washed once with phosphate-buffered saline containing 0.1% bovine serum albumin and incubated in minimal essential medium containing 1% heat-inactivated fetal bovine serum, and radiolabeled uridine (virus growth medium). The method for extraction and/or concentration of virus from infected cells or cell-free supernatent fluids was also previously described (8). Methods for purification of reovirus type 3 from infected L cells were the same as those used for RV14 (8).

Isopycnic centrifugation of viruses in cesium chloride gradients. The method used was described in detail previously (8). Briefly, viruses were centrifuged in CsCl solutions prepared in either TST buffer (0.2% tryptose-phosphate broth, 0.1 M NaCl and 0.05 M Tris, pH 7.4) or in TPBT buffer (as TST buffer, except Tris was at 0.01 M, pH 8.5). Similar results were obtained using either buffer. Centrifugation was at 33,000 rpm in the Spinco SW50.1 rotor for 24 h at 4 C. The density of every fourth fraction was immediately determined using a Bausch and Lomb Abbe-3L refractometer. Radioactivity and infectivity assays on each fraction were carried out as previously described (8).

Electron microscopy. Particles from the two bands of infectious virus were negatively stained with 2% uranyl acetate (pH 4.2) on formar and carbon-coated 400-mesh grids. They were photographed in a Philips EM-300 electron microscope at an original magnification of 70,000 diameters.

Chemicals. [5-^{3}H]Uridine (21.7 to 30.0 Ci/mM) and [2-^{14}C]uridine (55.6 mCi/mM) were purchased from New England Nuclear Co. (Boston, Mass.). CsCl was purchased from Sargent-Welch Scientific Co. (Anaheim, Calif.), Mallinckrodt Chemical Works (St. Louis, Mo.), or Schwarz/Mann Chemicals (Orangeburg, N.Y.). Freon (Precision Cleaning Agent, E. I. du Pont de Nemours and Co., Wilmington, Del.) was a gift from R. Z. Lockart, Jr. Antiserum against RV14 was a gift from V. V. Hamparian, Ohio Sate University, College of Medicine, Columbus. Pancreatic ribonuclease deoxyribonuclease, α-amylase and α-chymotrypsin were purchased from Worthington Biochemical Corp. (Freehold, N.J.). Trypsin was purchased from Flow Laboratories (Anaheim, Calif.).

RESULTS

Detection of two bands of RV14 in CsCl gradients. Extracts of HeLa cells that had been challenged with RV14 were mixed with solid CsCl and centrifuged to equilibrium as described in Materials and Methods. Assays of each gradient fraction for infectious RV14 has consistently revealed the presence of two bands of infectious RV14 (Fig. 1). The major band of infectivity had an average buoyant density of 1.411 g/ml and was coincident in position with the single band of radioactivity. The minor band of infectivity had an average buoyant density of 1.391 g/ml and contained less than 5% of the infectivity present in the major band. Virus in the minor band was never detected by radioactivity measurements and could only be detected by infectivity assays of the gradient fractions. Hereafter, the more dense band of infectious RV14 will be denoted as H virus and the less dense band of infectious RV14 will be denoted as L virus. The buoyant density of L virus in CsCl was further established by the following experiment. Infected L cells containing unlabeled reovirus type 3 were mixed with RV14-infected HeLa cells which had been incubated with [^{14}C]uridine to label RV14 and both viruses were simultaneously extracted. Isopycnic centrifugation in CsCl (Fig. 2) showed that reovirus type 3 and RV14 L virus exhibited identical buoyant densities of 1.380 g/ml, whereas the RV14 H virus had a buoyant density of 1.401 g/ml at peak coincident [^{14}C]uridine and infectivity values. This buoyant density value for reovirus type 3 is in agreement with data published in the literature wherein a similar purification procedure was used (1, 16). Average values from 14 experiments for buoyant densities of virus in the two bands of infectious RV14 in CsCl gradients were 1.409 $\pm$ 0.007 and 1.386 $\pm$ 0.004 g/ml for H and L virus, respectively.

The relative proportion of H and L virus extracted from infected cell cultures was estimated from infectivity titers of the peak fractions of each virus band. Separation of the two bands of infectious virus was not sufficient (Fig. 1 and 2) to permit the summing of infectivity titers in each fraction belonging to a specific band. The results of five experiments which were selected to show the range of proportion of infectivity in H/L virus bands are presented in Table 1. Infectivity titers in the L-virus peak fraction were 3.3 to 12.8% of the titers in the peak fraction of the H-virus band establishing that the majority of infectious virus extracted from infected cells was of H-virus type. Within the range of 2.50 $\times$ 10^{8} to 1.0 $\times$ 10^{10} cells

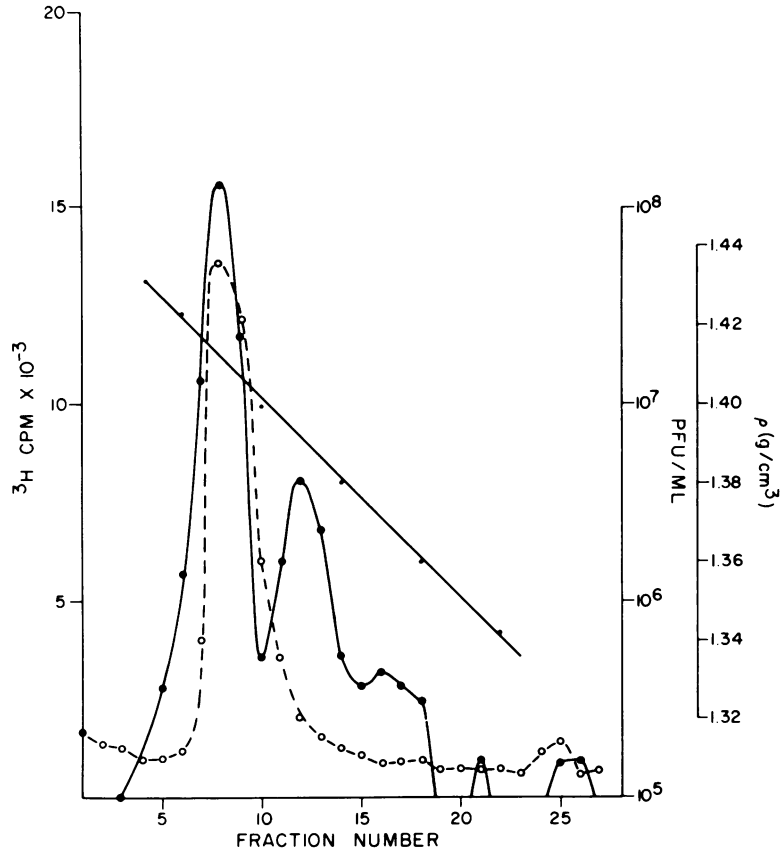

FIG. 1. *Isopycnic centrifugation of RV14 in CsCl gradients. HeLa cell cultures were challenged with RV14 and subsequently incubated in minimal essential medium containing [^{3}H]uridine. The cells were processed by the virus purification procedure and concentrated virus was centrifuged into a CsCl gradient as described in Materials and Methods. Each fraction was assayed for trichloroacetic acid-insoluble counts in [^{3}H]uridine* (○) *and PFU/ml of RV14* (●). *Density of every fourth fraction* (·) *was measured by refractometry.*

extracted per experiment, there were no correlations between number of cells extracted and extent of separation of H- and L-virus bands or proportion of infectivity in H/L viruses.

Several different conditions and procedures were used in these studies to determine if the presence of either band of virus was attributable to techniques used in the study. Both H- and L-virus bands were detected in CsCl gradients, using CsCl from three different commercial sources, including optically pure CsCl (Schwartz/Mann Chemicals). Two peaks of infectivity were present in CsCl gradients of virus prepared from either KB or HeLa cell cultures challenged with RV14, from either type of cell culture which was processed immediately upon harvest for purified virus or from either type of infected cell cultures that had been stored at −20 C for several weeks. Similarly, two bands of infectious virus were found if virus was centrifuged onto dense cushions of either 60% sucrose in TST buffer or CsCl at 1.5 g/ml density in TST buffer before mixture of the resulting interphase virus-containing fluids with solid CsCl and then subjected to isopycnic centrifugation. Centrifugation of virus-containing fluids, removed from dense CsCl cushions, into a preformed CsCl gradient (1.35 to 1.40 g/ml) in TST buffer for 10 h at 33,000 rpm in an SW50.1 rotor at 4 C also permitted detection of both H- and L-virus bands. However, in two experiments, only H virus was detected when virus particles were pelleted (100,000 × *g* for 2 h at 4 C) from virus-containing fluids removed from dense CsCl cushions and centrifuged into a preformed CsCl gradient as just described. This latter result was one of the earliest findings which suggested that L virus was labile under certain conditions; additional data on this point will be presented subsequently in Results.

Contamination of RV14 stocks by other rhinovirus serotypes. The concern that our

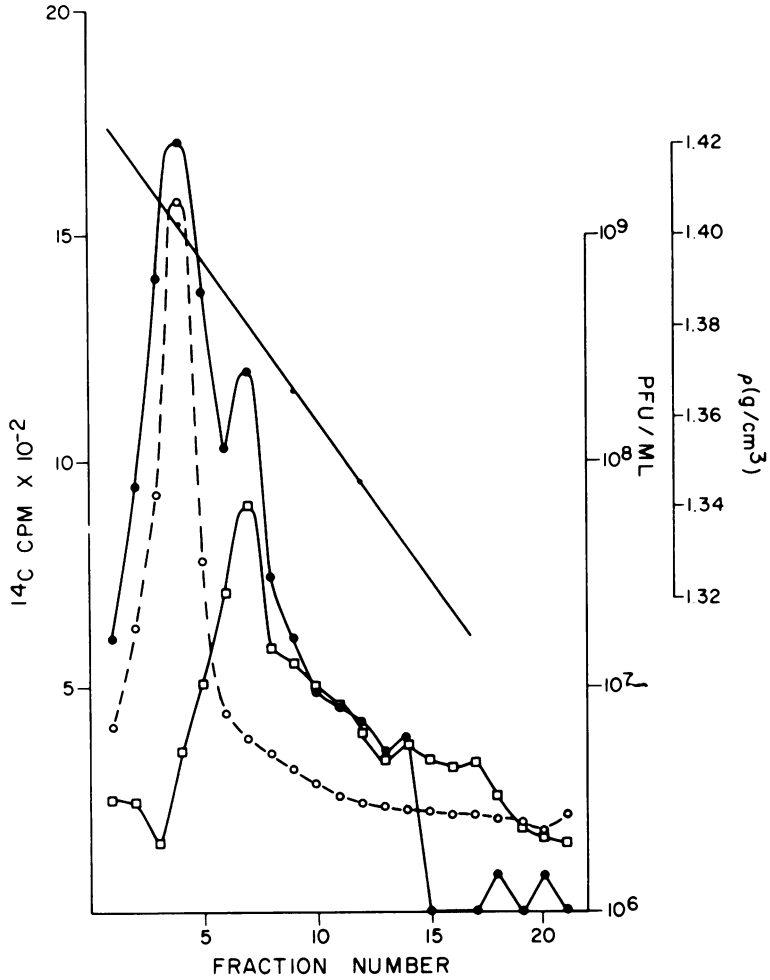

FIG. 2. *Cocentrifugation of RV14 and reovirus type 3 in CsCl gradients. L cells infected with reovirus type 3 were mixed with HeLa cells infected with RV14 and incubated with [^{14}C]uridine. The mixed cells were simultaneously processed by the virus purification procedure described in Materials and Methods. An aliquot of the solution containing both partially purified viruses was centrifuged into a single CsCl gradient using methods applied in the experiment shown in Fig. 1. Fractions were assayed for trichloroacetic acid-insoluble counts in ^{14}C in RV14 virus particles* (○), *PFU/ml of RV14* (●) *and PFU/ml of reovirus type 3* (□). *Density of every fourth fraction* (·) *was measured as in Fig. 1.*

stock virus preparations were contaminated by another rhinovirus serotype was dispelled by finding that (i) L- and H-virus bands were detected in virus prepared in either KB or HeLa cells using stock virus that originated from a plaque isolate that had undergone three successive plaque isolations; (ii) the plaque morphologies of L and H viruses were identical; (iii) RV14-monospecific antiserum neutralized infectivity, as measured by plaque assay, of both L and H viruses by 99.96% or greater.

Biophysical and biochemical differentiation between H and L viruses. Electron microscopic studies of H and L viruses were conducted to determine if aggregation of coreless and complete particles could account for infectivity in the L-virus band (an early hypothesis [27]) or if particles in the two bands were of different sizes. Electron photomicrographs of virus particles from H- and L-virus bands are shown in Fig. 3A and B. Virus particles of similar sizes were found in both bands. Average diameters (± average mean deviation) for single H- and L-virus particles were 35.5 ± 0.12 nm (101 particles measured) and 35.2 ± 0.31 nm (44 particles measured), respectively. Aggregates of virus particles were rarely found in the L-virus band, and therefore, coreless particles do not

have a significant role in formation of the L-virus band. An amorphous fibrous material was attached to most particles observed in the L-virus band. The composition of this fibrous material remains unknown, however, attachment of this material to the virus particles via protein linkage is suggested by results of the following experiment. Before the final step of banding of CsCl, a preparation of concentrated virus was divided into four aliquots. Each aliquot was incubated for 30 min at 37 C with one of the following mixtures of enzymes: (i) pancreatic ribonuclease (25 μg/ml) and deoxyribonuclease (25 μg/ml); (ii) α-chymotrypsin (50 μg/ml) and trypsin (50 μg/ml); (iii) α-amylase (50 mg/ml), or (iv) no enzymes. After the incubation period, each virus preparation was diluted 1:10 with TST buffer and mixed with solid CsCl to 1.385 g/ml and centrifuged for 24 h at 33,000 rpm at 4 C. Each fraction in these gradients was assayed for infectivity. Two bands of infectivity with buoyant densities of approximately 1.410 and 1.382 g/ml were found

TABLE 1. *Comparison of infectivity titers in H- and L-virus bands*[a]

Expt no.	H virus		L virus		Ratio of infectivity (L virus/H virus)
	Density (g/ml)	Infectivity (PFU/ml × 10^{-7})	Density (g/ml)	Infectivity (PFU/ml × 10^{-7})	
1	1.412	12	1.390	0.38	0.033
2	1.405	720	1.383	31	0.043
3	1.402	240	1.380	22	0.092
4	1.413	100	1.390	11	0.110
5	1.406	140	1.382	18	0.128

[a] Density and infectivity values are reported for the peak fraction of each band of infectious virus.

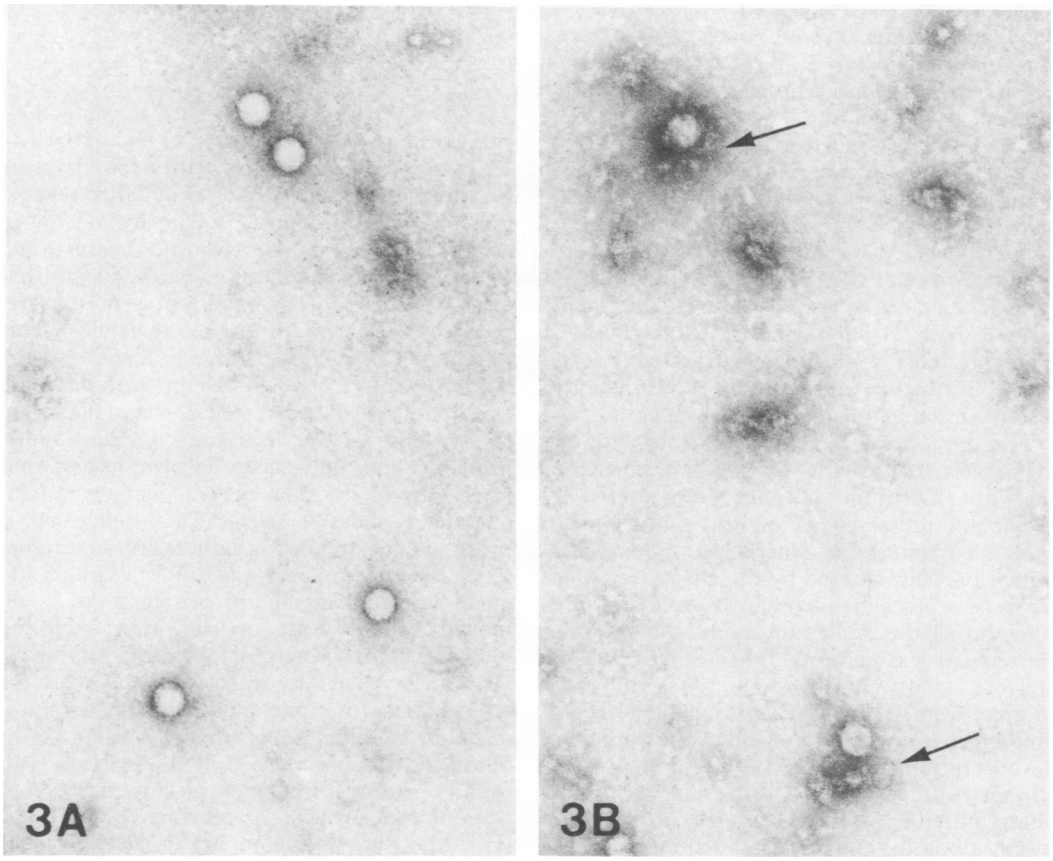

FIG. 3. *Electron photomicrographs of H and L virus. HeLa cells were challenged with RV14 and the cell culture was harvested at 16 h pi. Virus was extracted from the infected cells and centrifuged into CsCl. Fractions of the CsCl gradient were assayed for infectivity and two fractions were prepared for electron microscopy. (A) H-virus particles. (B) L-virus particles. Note fibrous material at arrows. Print magnification at ×121,000.*

in three of the gradients. Infectivity corresponding only to H virus was detected in the gradient into which the chymotrypsin- and trypsin-treated virus aliquot was centrifuged. Failure to detect the L-virus band after proteolytic enzyme treatment of the virus suggests that protein serves to attach the fibrous material to L-virus particles. Subsequent experiments suggested a labile attachment of virus to the fibrous material. L-virus band virus subjected to a second isopycnic centrifugation in a CsCl gradient resulted in recovery of greater than 75% of infectivity in the gradient at an average buoyant density of approximately 1.41 g/ml in two experiments. These experiments suggest that the fibrous material is responsible for altering the buoyant density of some virus particles and it is attached to the particles via labile bonds which contain protein.

Origin of L virus. All previous experiments had been carried out with virus obtained from infected cells (cell-associated virus). In view of the fact that the buoyant density of L virus appeared to reflect a cell-directed interaction of virions and less dense fibrous material, it was of interest to determine if virus purified from cell-free fluids of infected cells contained both L and H viruses. In two experiments, virus purified from cell-free supernatant fluids contained only a single infectious band of RV14 at a buoyant density in CsCl gradients which corresponded to that for H virus. Cell-associated virus in each of the two respective experiments yielded both H- and L-virus bands in CsCl gradients. This result further suggested that the virus purification procedure did not inherently lead to an artifactual production of L virus.

The data thus far are compatible with the hypothesis that L virus represents a portion of the virus inoculum that has been altered by metabolic processes during the virus growth cycle. To determine whether L virus represented particles altered by the adsorption process or by a process(es) which occurred at a later time during the replication cycle, the following experiment was performed. Monolayer cultures of HeLa cells (approximately 100×10^6 cells/sample) were challenged with purified [^{3}H]uridine-labeled RV14. At the end of the 2-h period for adsorption, the inoculum was removed and all cell cultures were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin, and virus growth medium was added. One cell sample was incubated for 1 additional h, harvested at 3 h postinoculation (pi), and immediately taken through the virus extraction procedure. The second cell sample was incubated for an additional 10 h at 34.5 C before harvest and immediate processing for extraction of virus particles. Virus particle-containing fluids were mixed with solid CsCl and centrifuged to equilibrium. Each fraction of the gradients was then assayed for trichloroacetic acid-insoluble counts in ^{3}H and for infectivity. The results are presented in Fig. 4. L-virus was not extracted in detectable quantities from cells harvested at 3 h pi. Thus, formation of this class of virus particles requires metabolic processes which take place after adsorption of most particles has occurred (9). Both H and L viruses were detected in cells harvested at 12 h pi, and although the H-virus band was clearly detected by radioactivity assay, some radioactivity was found in the region of the L-virus band. These results suggest that attachment of fibrous material to H virus, resulting in conversion of H virus to L virus, occurred at an intracellular site between 3 and 12 h pi. The reversible alteration of H and L virus is therefore not merely a consequence of the adsorption process.

DISCUSSION

The detection of two bands of infectious rhinovirus in CsCl gradients is not limited to RV14, as similar results have been found during isopycnic centrifugation of rhinovirus types 2 and 5 in CsCl gradients (27). The initial hypothesis for explaining our results (Gauntt and Sauck, unpublished data reference 27) was that virus in the less dense band represented aggregates of virions and coreless particles. This hypothesis is clearly incorrect in view of the data presented herein. The present data are compatible with the hypothesis that a portion of the virions in the inoculum is sequestered, probably internally, after adsorption and that the sequestered virions remain coated throughout the replication cycle. This conclusion is based on the following data. The sequestered particles are similar in size to RV14 virions and once the attached amorphous material is removed from L-virus particles, they exhibit a buoyant density in CsCl gradients similar to virions. L virions are infectious, and therefore are different from particulate components produced by acidification of rhinovirions (14, 22) or obtained after interaction of rhinovirions with cells (17, 18). L-virus particles apparently contain similar antigenic determinants as virions (H virus), based on comparable levels of neutralization of PFU by antiserum directed against RV14. L virus is not detected in supernatant fluids harvested from RV14-infected cells after one complete cycle of replication nor from cells harvested immediately after the virus

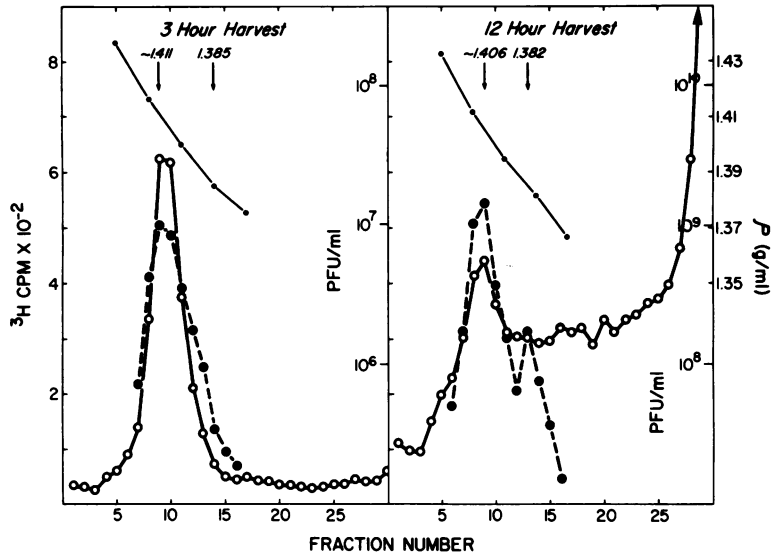

FIG. 4. *Isopycnic centrifugation in CsCl gradients of RV14 extracted from HeLa cells challenged with labeled RV14 and harvested after the adsorption period or at completion of one growth cycle. HeLa cells were challenged with a multiplicity of 5 to 10 PFU/cell of [^{3}H]uridine-labeled RV14 (278 PFU/counts per min) and incubated at 34.5 C for 2 h. After removal of the inoculum, the cells were washed twice with minimal essential medium and incubated in virus growth medium for either one or 10 additional h and processed to yield RV14 virus particles. Symbols:* (○) *trichloroacetic acid-precipitable ^{3}H counts (counts/min);* (●) *infectivity (PFU/ml), and* (·) *density (g/ml).*

adsorption period, suggesting that L virus does not originate as an artifact of our procedure or techniques. In support of the latter statement, L virus is also detected in infected KB and L-132 cells and is found in freshly harvested infected cells or infected cells stored for as long as 3 weeks at −90 C (Gauntt, Griffith, and Sauck, unpublished data). Additionally, mengovirus and coxsackie B3 virus produced in the strain of HeLa cells used in this study exhibit only one band of infectious virus in CsCl gradients (Gauntt, unpublished data).

The nature of the fibrous material attached to L virus is unknown; however, the results presented herein suggest that attachment of the fibrous material to virions is via labile protein linkage. A thread-like material of a similarly amorphous nature to that associated with L-virus particles was found associated with poliovirus particles that cosedimented with the viral RNA replication complex (3). Whether the thread-like material or fibrous material in the present study represents excess precursor material which normally participates in formation of progeny virus particles (3) or represents products arising from intracellular degradation of virus particles (24, 32) is unknown. However, data presented in this study suggests that active production of virus must be in progress as attachment of amphorous material to virus particles does not occur during the adsorption period.

The fact that a portion of the inoculum (3 to 13%) is taken up but does not participate in replication is not unique to the RV14-HeLa cell system, as it is well known that from 1.5 to 25% of poliovirus particles (7, 19) and from 5 to 20% of encephalomyocarditis virus particles (11) in challenge inocula do not undergo eclipse and remain within the cell as intact virus particles. An explanation for this phenomenon is not at hand. Either some cells lack viral uncoating enzymes (5) or, alternatively, uncoating of virions may be a function of virion-bound proteinases (12) and virus particles which lack these enzymes do not participate in viral-replicative processes.

The persistence of picornavirus particles in cells is not a general characteristic of this group of viruses, however, a few studies have been published which suggest that this phenomenon does occur. Although there have been several reports on the presence of picornavirus-like particles in muscle tissue of human patients with myositis and polymyositis, one recent report has identified coxsackie A9 virus particles in skeletal muscles of a patient with chronic myopathy (29). Foot-and-mouth disease virus has been shown to establish a persistent infection of cells of the bovine pharynx and in some

cases, in absence of seroconversion to viral antigens (28, 31). Persistence of some picornaviruses in cells may be an important factor in some diseases caused by these viruses.

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