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Density Gradient Centrifugation Studies on Lymphocytic Choriomeningitis Virus and on Viral Ribonucleic Acid

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Lymphocytic choriomeningitis (LCM) virus, Traub strain, was purified from BHK-21 tissue culture medium. The virus was then analyzed by equilibrium centrifugation and rate zonal centrifugation in sucrose gradients. A buoyant density in sucrose of 1.18 g/ml was found and the $S_{20,w}$ value was estimated to be about 470 to 500*S*. Furthermore, the ^{3}H-uridine-labeled ribonucleic acid (RNA) from virus was extracted from LCM virus and analyzed by rate zonal centrifugation. Two major and one minor single-stranded RNA components were found with sedimentation coefficients of 28, 22, and 18*S*.

Although lymphocytic choriomeningitis (LCM) virus has been known for more than 30 years, most work on this virus has been ascribed to problems concerning the carrier state in mice and cells in tissue culture.

Recently, the first electron microscopic observation of LCM virus was published (6), which described the virus as pleomorphic, ranging in size from 50 to 300 nm. Furthermore, the virus was reported to contain from one to eight ribonuclease-sensitive granules. The virus particles were formed by budding from the cell membrane and appeared to have spikes. It has been reported also that the virus is ether-sensitive (27) and therefore contains lipid.

The present investigation concerns the physical behavior of LCM virus in sucrose gradients, and the results seem to indicate that LCM virus is not so heterogenous when centrifuged to equilibrium in sucrose as when it was centrifuged in tartrate gradients (5, 26). Rate zonal centrifugation of LCM virus described in this paper seems to confirm the above mentioned electron microscopic observations which described that the virus was heterogenous in size.

Various chemical inhibitors have been used to show that LCM virus belongs to the ribonucleic acid (RNA) viruses (3, 4, 15, 28, 29; J. L. Barlow, S. M. Cohen, I. Triandaphilli, 1965, Fed. Proc. **24**:319), but no direct evidence has been available. By analysis in sucrose gradients of the RNA liberated from purified virus, we have found direct evidence for three RNA components of the virus; it is, therefore, presumed that these components represent several pieces of the viral genome.

MATERIALS AND METHODS

Materials. Uridine-5-^3H (>15,000 mCi/mmole) and uridine-2-^{14}C (>50 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, England. Bovine pancreatic ribonuclease 5× crystallized was obtained from Sigma Chemical Co. Albumin (fraction V) B grade and 7*S* human globulin were obtained from Calbiochem. 2,5-Diphenyloxazole (PPO) and dimethyl 1,4-bis-2-(S-phenyloxazolyl)-benzene (POPOP) were obtained from Beckman Instruments, Inc.

Virus. The LCM virus, Traub strain, was isolated from AKA mouse spleens and was then passaged three times in BHK-21 cells which were grown in suspension. The cell-free medium was used as a stock virus source in all experiments, and the titer was about $10^{6.5}$ LD_{50}/0.03 ml. Virus was stored at −70 C.

Tissue culture. BHK-21 cells were cultivated in suspension at 37 C, with a concentration of 2×10^5 cells per ml in 1-liter flasks. The medium used was BHK-21 medium, MacPherson and Stoker (21), supplemented with 10% Tryptose phosphate broth and 10% fetal calf serum. For preparation of virus stocks, 5% heat-inactivated fetal calf serum was used.

When BHK-21 cells were used for virus infection, the initial cell concentration was 9×10^5 cells per ml, and the medium was supplemented with 0.5% heat-inactivated fetal calf serum. The cells suspended in a total volume of 240 ml were infected with 32 ml of stock LCM virus, and at the same time the cells were labeled with 2.0 ml of ^{3}H-uridine (2 mCi). The cells were diluted to 3×10^5 cells per ml 90 min later and supplemented with 1% heat-inactivated fetal calf serum.

Control experiments were always performed simultaneously. In these experiments, one culture of noninfected uridine-labeled cells and another culture of infected nonlabeled cells were employed. The cell-free

medium from these two cultures was mixed as described below when the cultures were harvested. In the culture with no radioactive labeling, cells were infected with the same ratio between virus and cells as described above, but the final volume after dilution was 200 ml. The noninfected, 200-ml control culture was labeled with 0.5 ml of ^{3}H-uridine (0.5 mCi).

Unlabeled uridine (25 mg) was added to the radioactive-labeled cultures 24 to 48 hr after infection; after 10 min, the cultures were harvested by centrifugation in a Sorvall HG4 rotor at 5,000 rev/min for 5 min (6,975 × *g*) at 4 C. The supernatant fluids were isolated and used for virus purification. Cells in all cultures were tested for virus antigen by the fluorescent antibody method (24), and the virus-containing media were used only in the experiments if the cells were almost 100% infected. Noninfected cells never showed any trace of fluorescent virus antigen.

Virus purification. The supernatant fluids described above were cooled to 0 C, and virus was precipitated by the slow addition of an equal volume of a cold, saturated, neutralized ammonium sulfate solution. The temperature never exceeded 3 C during precipitation. After the addition of ammonium sulfate, the virus was allowed to precipitate further for 30 min in the cold. After precipitation, the solutions were centrifuged in a Sorvall HG4 rotor at 5,000 rev/min (6,975 × *g*) for 10 min at 0 C. The isolated precipitates were washed in 24 ml of 50% saturated ammonium sulfate in 0.01 M tris(hydroxymethyl)aminomethane (Tris), HCl, 0.1 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), *p*H 7.4 (TES buffer). The washed precipitates were centrifuged again in a Sorvall HB4 rotor at 3,000 rev/min (1,465 × *g*) for 5 min at 0 C, and the precipitates were then dissolved in 10 ml of cold TES buffer containing 5 mg of unlabeled uridine and 1.0 ml of 1% bovine serum albumin. The dissolved virus was then centrifuged in a Sorvall HB4 rotor at 10,000 rev/min (16,300 × *g*) for 10 min at 0 C. The supernatants were isolated and diluted to 20 ml with more cold TES buffer. A 19-ml volume of the virus material was then further purified by centrifugation on a double cushion, consisting of a bottom layer made of 5 ml of 60% (w/v) sucrose mixed with 15% CsCl (w/v), and an upper layer made of 5 ml of 15% (w/v) sucrose. Centrifugation was performed in a Spinco SW-25.1 rotor at 22,000 rev/min for 1 hr at 5 C. After centrifugation, a band of virus material was visible between the two sucrose layers. The virus band, in a volume of about 1 ml, was isolated by collection of drops from a needle inserted in the bottom of the tube. To the virus band was added 0.1 volume of 1% bovine serum albumin, and the virus material was dialyzed overnight against 200 ml of cold TES buffer (*p*H 8.4). The dialyzed virus material in a volume of about 1 ml was then centrifuged to density equilibrium after it was layered over a 28-ml 15 to 65% preformed sucrose gradient in TES buffer. The centrifugation was performed in a Spinco SW-25.1 rotor with a rotor speed of 22,000 rev/min for 16 hr at 5 C. After centrifugation, about 28 fractions were obtained by collecting drops from the bottom of the tube. The radioactive peak of virus was localized by measuring the radioactivity of 50 μliters of the fractions. When the peak was located, the three or four top fractions were pooled. Human 7*S* globulin (1 mg) was added as a coprecipitant, and the pool was precipitated for 30 min in the cold by addition of an equal volume of saturated ammonium sulfate solution. The precipitate was collected after centrifugation in a Sorvall HB4 rotor at 6,000 rev/min (5,860 × *g*) for 10 min at 0 C. The precipitate was finally dissolved in 0.5 or 1.0 ml of cold TES buffer, and this purified virus solution could be stored at −20 C.

Isolation of RNA from cells. BHK-21 cells (200 ml) grown in suspension with a cell concentration of 3×10^5 cells per ml were labeled for 24 hr with 50 μliters of ^{14}C-uridine (500 μCi). The cells were isolated by low speed centrifugation, and the RNA was isolated by the hot phenol extraction procedure as described by Scherrer and Darnell (16, 37).

Isolation of RNA from virus. Purified virus was disrupted by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1% SDS. ^{14}C-uridine-labeled host RNA was added as a reference marker, and the solution was then allowed to stand for 10 min at room temperature before being layered on a sucrose gradient.

Ribonuclease sensitivity test. Viral RNA was tested for ribonuclease sensitivity by addition of bovine pancreatic ribonuclease to a final concentration of 1 μg/ml. The solutions were then incubated at 37 C for 30 min, and acid-precipitable counts were determined.

Virus assay. Virus titrations were performed by intracerebral inoculation of serial 10-fold dilutions of virus in 12- to 14-g Swiss mice. Virus (0.03 ml) was injected in groups of eight mice for each dilution. The titration end points were calculated by the method of Kärber (18) and expressed as log LD_{50}/0.03 ml.

Radioactivity determinations. Acid-precipitable radioactivity was determined in all samples by addition of 0.1 mg of bovine serum albumin followed by trichloroacetic acid to a final concentration of 5%. The samples were kept at 0 C for 30 min, and the precipitates were collected on Sartorious nitrocellulose membrane filters (0.45 μm), which were washed with 15 to 20 ml of cold 5% trichloroacetic acid, dried overnight at 37 C or at 60 C for 60 min, and then counted in 5-ml scintillation solutions consisting of 4 g/liter of PPO and 0.2 g/liter of dimethyl POPOP in toluene. Radioactivity was measured in a Beckman (model LS-250) liquid scintillation counter.

RESULTS

Equilibrium centrifugation of LCM virus. ^{3}H-uridine-labeled LCM virus was first precipitated with ammonium sulfate and was next centrifuged on a double sucrose cushion as described above. The semipurified, dialyzed virus material was then centrifuged to density equilibrium in a 15 to 65% preformed sucrose gradient as described in Fig. 1. After centrifugation in a Spinco SW-25.1 rotor at 22,000 rev/min for 16 hr at 5 C, the virus material was collected from the bottom of the tube. From the fractions collected, 100 μliters was isolated for

measurement of virus infectivity, and 100 μliters was used for the determination of sucrose density in a refractometer. The remaining volume of each fraction was precipitated with trichloroacetic acid for determination of radioactivity. About 100% of the counts were recovered.

The results from the equilibrium centrifugation in Fig. 1 showed that the virus material bands in a narrow zone, and it can be seen that virus infectivity and radioactivity coincide, indicating that all ^{3}H-uridine material has the density of infectious virus. The buoyant density of the LCM virus in sucrose is estimated to be 1.18 g/ml, which is the average value from several similar experiments.

The density of LCM virus in sucrose is close to the density value of about 1.16 to 1.17 g/ml in sucrose which was reported for the RNA tumor viruses (11, 32) and is also near the value of d = 1.19 g/ml which was reported for Sindbis virus (40).

Rate zonal centrifugation of LCM virus. Figure 2 shows the results of rate zonal centrifugation of purified ^{3}H-uridine-labeled LCM virus in a 5 to 20% sucrose gradient. Purified LCM virus was obtained by isolating the three or four top fractions after equilibrium centrifugation, as described above. The purified virus material in a volume of about 0.5 ml was layered on the top of a preformed 5 to 20% sucrose gradient and was then centrifuged in a Spinco SW-25.1 rotor at

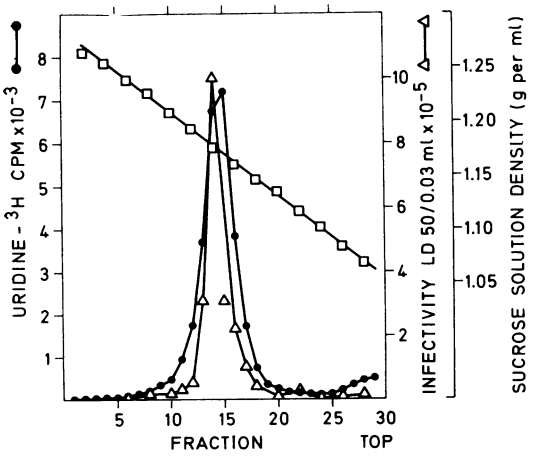

FIG. 1. *Equilibrium density gradient centrifugation of ^{3}H-uridine-labeled LCM virus. Virus material (0.8 ml) was layered on the top of a 28-ml preformed 15 to 65% (w/v) sucrose gradient containing 0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4. The virus was centrifuged in a Spinco SW-25.1 rotor at 22,000 rev/min at 5 C for 16 hr. Fractions were collected from the bottom of the tube. Symbols: (△) viral infectivity, (●) ^{3}H-uridine counts/min, and (□) solution density.*

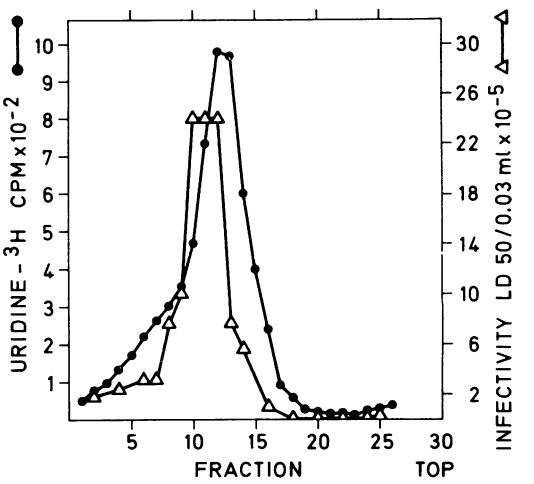

FIG. 2. *Rate zonal centrifugation of purified ^{3}H-uridine-labeled LCM virus. Purified virus (0.4 ml) was layered on top of a 28-ml preformed 5 to 20% (w/v) sucrose gradient containing 0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4. The virus was centrifuged in a Spinco SW- 25.1 rotor at 20,000 rev/min at 5 C for 75 min. Fractions were collected from the bottom of the tube. Symbols: (△) viral infectivity and (●) ^{3}H-uridine counts/min.*

20,000 rev/min for 75 min. There was no loss in the recovery of radioactive counts.

The LCM virus sedimented in one band, but this band showed a rather broad distribution of the virus material which indicates a heterogenous size of the virus. This finding confirms the results of Dalton et al. (6), who observed LCM virus by electron microscopy. Dalton et al. described the virus particles as being pleomorphic, with a size distribution from 50 to 300 nm.

Virus infectivity can be seen to follow the leading portion of the zone of ^{3}H-uridine-labeled material, indicating that not all of the ^{3}H-labeled material is infectious, or that the larger LCM virus particles are more complete and, therefore, more infectious. A similar discrepancy between virus infectivity and radioactive labeling has been observed with RSV labeled with ^{32}P (32) or influenza virus labeled with ^{32}P (12).

The $S_{20,w}$ value for the peak of radioactivity was calculated, using the tables outlined by McEwen (20), to be about 470S without the use of an internal marker. The S value for the peak of infectivity was calculated to be about 500S. In these calculations, corrections for acceleration and deceleration were made; when the tables were used, the density of the LCM virus was assumed to be 1.20 g/ml. The density of 1.20 g/ml was chosen because this value in the tables was the closest to our observed density of d = 1.18 g/ml.

Sedimentation characteristics of LCM virus RNA. The nucleic acid from purified LCM virus labeled with ^{3}H-uridine was liberated from the virus by treatment with SDS, by using a final concentration of 1% SDS, as described above. The liberated viral RNA was analyzed by velocity sedimentation in a 5 to 20% sucrose gradient (Fig. 3), and about 90% of the radioactive counts were recovered. The sucrose was dissolved in TES buffer, and the RNA was centrifuged for 15 hr in a Spinco SW-25.1 rotor at 20,000 rev/min. ^{14}C-uridine-labeled BHK-21 cell RNA was used as a sedimentation marker. It should be noted that the hot phenol extraction procedure used to prepare the marker RNA displaced the 28*S* host RNA to a lower value when the RNA was analyzed on a sucrose gradient. This was due to the liberation of a 7*S* component from the 28*S* ribosomal RNA (13, 25, 31). Consequently, the *S* value for the 28*S* host RNA is quoted to be 26*S*.

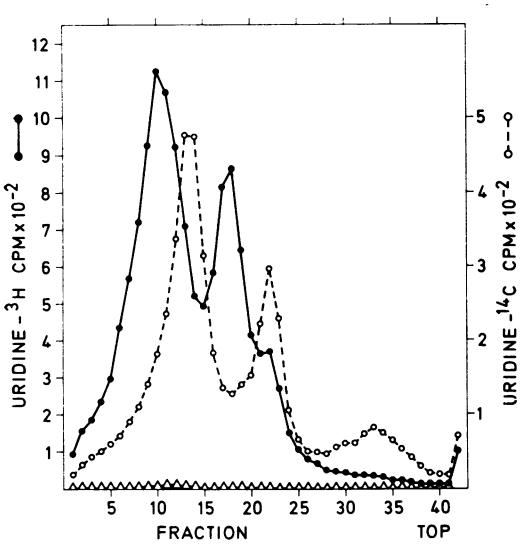

FIG. 3. *Sucrose gradient centrifugation of ^{3}H-uridine-labeled RNA from purified LCM virus with ^{14}C-uridine-labeled BHK-21 cell RNA markers. A mixture of 250 μliters of SDS-disrupted virus and BHK-21 cell RNA was layered on the top of a 28-ml preformed 5 to 20% (w/v) sucrose gradient containing 0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4. Centrifugation was at 20,000 rev/min, at 11 C for 15 hr in a Spinco SW-25.1 rotor. Fractions were collected from the bottom of the tube. A 200 μliter amount of each fraction was incubated for 30 min with pancreatic ribonuclease with a final concentration of 1 μg/ml at 37 C, before determination of trichloroacetic acid-precipitable radioactivity. Symbols: (●) ^{3}H-uridine-labeled LCM RNA, (○) ^{14}C-uridine-labeled BHK-21 cell RNA, and (△) ^{3}H-uridine-labeled LCM RNA after ribonuclease treatment.*

It can be seen that exposure of the virus to 1% SDS results in a marked reduction in sedimentation rate of the ^{3}H-uridine-labeled material compared to untreated virus in Fig. 2. Two distinct peaks are present, and it is believed that they represent viral RNA. The $S_{20,w}$ value for the two RNA peaks was calculated to be 28*S* and 22*S*, by the method of Martin and Ames (22), using the 18*S* BHK-21 cell RNA as the marker. Furthermore, a shoulder of virus RNA can be seen in the 18*S* area. Applying Spirin's relation, $MW = 1{,}550 \times S^{2.1}$ (38), an approximate molecular weight of 1.7×10^6, 1.1×10^6, and 0.7×10^6 daltons can be calculated for the 28, 22, and 18*S*, respectively.

The different fractions were also tested for ribonuclease sensitivity. All ^{3}H-uridine and most of the ^{14}C-uridine RNA material was rendered acid soluble by pancreatic ribonuclease, indicating that all viral RNA molecules were single stranded.

The data shown in Fig. 3 indicate that the RNA species of LCM virus are single stranded. To confirm these results and to examine whether the LCM RNA peaks sediment differently from the host RNA under other centrifugation conditions, the sedimentation was examined in low ionic strength buffer. The dependence of sedimentation rate on salt concentration is well known for single-stranded RNA molecules (41). An example for this dependence has been reported for single stranded viral RNA from other lipid-containing RNA viruses (9, 10, 17, 36, 40).

The results of a centrifugation of LCM RNA in 0.001 M salt are shown in Fig. 4A. When the viral RNA was centrifuged under the same conditions but in 0.1 M salt (Fig. 4B), the sedimentation rate for the 28 and 22*S* viral RNA was reduced approximately 33 and 14%, respectively; the rate for the host cell ribosomal RNA molecules was reduced about 27%. The two large viral RNA peaks are now coinciding, and the 28*S* viral RNA, which before was separated from the host cell RNA, now sediments at the same position. This observation indicates that the 28 and 22*S* LCM RNA could be different from the host RNA and each other, as the relative position between viral and host RNA is changed by different conditions of sedimentation. The viral RNA peak, or shoulder, in the 18*S* area was reduced to the same degree as the 18*S* host RNA, so these two RNA species could not be differentiated by sedimentation in low salt buffer.

DISCUSSION

By analyzing the LCM RNA in sucrose gradients, we have observed two major and one minor single-stranded RNA components of the purified virus with sedimentation constants of 28, 22, and

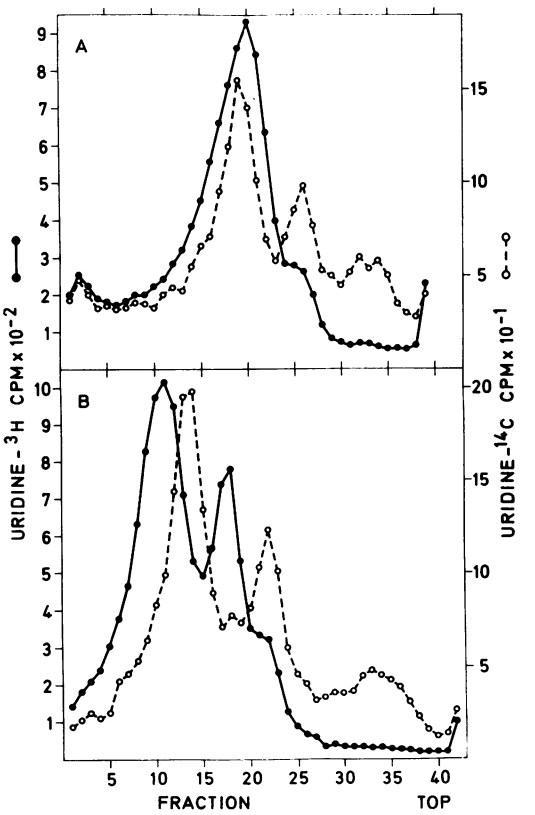

FIG. 4. *(A) Sucrose gradient centrifugation of ^{3}H-uridine-labeled RNA from purified LCM virus with ^{14}C-uridine-labeled BHK-21 cell RNA in low salt buffer. Centrifugation was performed as described in the legend of Fig. 3, except for the buffer used, which contained 0.001 M Tris, 0.001 M EDTA, pH 7.4. (B) An identical sample as described in (A) was centrifuged at the same time in 0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4, as a comparison to (A). Symbols: (●) ^{3}H-uridine-labeled LCM RNA, and (○) ^{14}C-uridine-labeled BHK-21 cell RNA.*

18*S*. The question now arises of whether all three components represent true intact components, when they are liberated from the virus, because most of the RNA viruses examined contained only one major molecule of viral RNA. RNA from RNA tumor viruses may also be heterogenous (1, 2, 9, 11, 14, 17, 36), but influenza virus is the only examined virus which, with a high degree of certainty, contains different RNA molecules (8, 10, 12, 19, 30).

To account for the observed heterogeneity of the LCM RNA, several explanations can be presented, but not all can be well defined by the present experiments. The possibility exists that the viral genome consists of a single piece of RNA with preferential breaking points; in this way, each component can be recovered reproducibly. The very gentle treatment of the LCM virus with SDS only, to free the viral RNA, would be an argument against breakdown of any viral RNA. The larger RNA pieces could also be aggregates of smaller components, although we used EDTA in the sucrose gradients to prevent such aggregates. A third possibility for finding several viral RNA components is that the LCM preparation consists of several classes of viruses, each containing one different molecule of RNA. We exclude this, because we did not find several distinct classes of virus after rate zonal centrifugation. If the peak of virus after rate zonal centrifugation was localized and separated into a left and a right part, and the RNA was isolated, then the same RNA composition as shown in Fig. 3 was found. This would not be expected if the virions of varying size contained different RNA genomes.

It is unfortunate that two of the viral RNA species sediment in the host 26 and 18*S* area, as one immediately will think of host RNA contamination. But we feel that viral 28 and 18*S* cannot be due to host RNA, which becomes associated with the virus during the purification procedure, because the virus preparation is not sensitive to pancreatic ribonuclease. Moreover, the reduction in sedimentation rate of the 28*S* viral RNA and the 26*S* host RNA, by sedimentation in low ionic strength buffer, was also different, which favors the idea that the viral and host RNA are different in configuration. Finally, the relative proportion of the viral RNA peaks was not altered significantly from preparation to preparation; this would probably be the case if one of the RNA peaks were an extraneous contaminant.

Further analyses are required to determine if there is ribosomal host cell RNA inside the virion. To the author's knowledge, this phenomenon has not been reported for any other RNA viruses, but it has been reported that host transfer RNA may contaminate the RNA isolated from avian myeloblastosis virus (14).

In considering whether our purified LCM preparation is contaminated with other RNA viruses originally contained in the stock LCM pool, we believe that this possibility is very remote. If the viral RNA is isolated from virus purified by rate zonal centrifugation or equilibrium centrifugation, then the same RNA pattern as seen in Fig. 3 is obtained. If a contaminating RNA virus is present, however, it would be required to have the same physical behavior as LCM virus. A contaminating RNA virus in the BHK-21 cells is also not likely, unless it is induced to grow by LCM virus, because the radioactive counts in the con-

trol virus bands never exceeded the test viral band by more than about 3%.

To reiterate, it seems unlikely that the different LCM RNA components are due to some kind of artifacts, and we presume that they may represent several RNA genomes of this virus. To emphasize this hypothesis, it can be noted that Dalton et al. (6) observed in the electron microscope several ribonuclease-sensitive granules within the LCM virus, and they suggested that the LCM virion may carry more than one copy of the genome. Our results indicate that these genomes may be different.

The buoyant density of LCM virus in sucrose was estimated to be $d = 1.18$ g/ml. This value is in agreement with the results of Camyre and Pfau (5), who reported the buoyant density of LCM strain WCP in potassium tartrate gradient to be 1.24, 1.20, and 1.17 g/ml with 90% of the infectivity associated with the band having a density of 1.17 g/ml if 20 to 45% gradients were used.

Recently, the LCM virus has been shown to be serologically and morphologically related to other RNA viruses (23, 35), and it has been suggested that the name arenoviruses should be given to viruses in this new taxonomic group (34). This proposal is very well supported by the present experiments, as the LCM RNA sediments very differently compared to the RNA of other known RNA viruses. We feel that the criteria for placing a virus in the arenovirus group should also include that the virus have a buoyant density in sucrose of about 1.18 g/ml and that the RNA should be single stranded.

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