JOURNAL OF VIROLOGY, May 1968, p. 409-414
Vol. 2, No. 5

Density Gradient Centrifugation of Rubella Virus

ROBERT M. McCOMBS AND WILLIAM E. RAWLS
Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas 77025

Received for publication 2 February 1968

Rubella virus was centrifuged in sucrose density gradients. One of two densities could be ascribed to the virus, depending upon the suspending medium used. The virus was found at a density of 1.16 g/cm³ after centrifugation for 18 hr in sucrose gradients prepared in distilled water. By contrast, when the sucrose gradients were prepared in tris(hydroxymethyl)aminomethane (Tris)buffer containing ethylenediaminetetraacetic acid (EDTA), the virus was found at a denisty of 1.18 g/cm³ after 18 hr of centrifugation. The virus banded at this higher density after only 2 hr of centrifugation when pretreated by overnight incubation in the Tris-EDTA buffer. A kinetic study showed that, in sucrose gradients containing this buffer, the virus gradually migrated as a single peak of infectivity from a density of 1.16 g/cm³ after 2 hr of centrifugation to the higher 1.18 g/cm³ density after 18 hr. The density change was shown to be reversible; after the removal of the Tris-EDTA buffer, rebanding of virus harvested at the heavy density resulted in its banding at the lower 1.16 g/cm³ density. The data indicate that density change could not be explained on the basis of the loss of some component from the virus or on the basis of the failure of the virus to reach equilibrium. However, it is possible that the two densities observed were a reflection of the existence of rubella virus in different hydration states in the presence and absence of Tris buffer containing EDTA.

Recent studies of the clinical manifestations and the various complications arising from congenital rubella virus infection (17, 18) have emphasized the necessity for the complete characterization of this virus. Rubella has been characterized as an ether-sensitive ribonucleic acid-containing virus (15). However, studies of other biophysical parameters of this virus have not been uniformly successful. Density gradient centrifugation studies of rubella have generally reported an unusually low density of approximately 1.08 g/cm³ (*see* Table 3). These studies may have been hampered, in part, by low infectivity titers and difficult assays. More recent studies (3, 7, 23), however, indicate that the density of rubella virus is significantly higher than that reported previously, and that this virus appears to be more closely related in this respect to other lipid-containing, membrane-bound viruses (12, 14, 21). The present communication describes a significantly higher density for rubella virus in sucrose gradients than has been reported previously.

MATERIALS AND METHODS

Cell cultures. Serially propagated human embryonic lung cells (12) and continuous cell lines of green monkey kidney, BSC-1 (5), and hamster embryo fibroblast cells, BHK_{21} (25), were used in this study. These cells were grown in Eagle's medium with 10% fetal bovine serum and 0.075% sodium bicarbonate (for cells in stoppered vessels) or 0.225% sodium bicarbonate (for cells in petri dishes in a 5% CO_2 atmosphere). The cells were maintained in the same medium containing 2% fetal bovine serum. Primary cultures of African green monkey kidney cells (GMK) were prepared as described by Melnick (9).

Viruses. The rubella virus strains used in this study were obtained from the fluid of carrier cultures derived from thyroid (R-1) and lung (R-3) tissue of two different infants with congenital rubella (17). Virus stocks were prepared by inoculating BHK_{21} monolayers with 1 ml containing $10^{4.5}$ inhibitory dose 50 (IND_{50}) of rubella virus. After 1 hr of adsorption at 37 C, the cells were covered with maintenance medium, which was changed at 3- to 4-day intervals. The virus-containing culture fluid was harvested after the seventh day. Virus stocks were also prepared in human embryonic lung by a technique similar to that described by Plotkin, Boue, and Boue (16).

Newcastle disease virus (NDV; California strain, American Type Culture Collection) was passaged in 11-day-old embryonated eggs, and the allantoic fluid was harvested after 48 hr. The resulting virus suspension contained 3×10^8 plaque-forming units (PFU)/ml when assayed in chick embryo fibroblast monolayers. Echovirus 11 (Gregory) was obtained from the World Health Organization International Reference Centre for Enteroviruses, Houston, Texas, and working virus stocks were prepared in GMK cells.

Virus assay. Rubella virus was assayed with a

modification of the hemadsorption-negative plaque test described by Marcus and Carver (8). Monolayers of BSC-1 cells were grown in 60-mm plastic petri dishes. The medium was removed, and 0.2 ml of virus inoculum was added to the petri dishes. The virus was allowed to adsorb for 1 hr at 37 C, and the monolayers were then covered with 4 ml of maintenance medium. After 2 days of incubation at 37 C, 0.2 ml of a 1:4 dilution of the NDV stock was added to the plates. The plates were incubated for an additional 16 to 20 hr; then the medium was removed and 2 ml of a 0.5% suspension of sheep red blood cells was added. After 20 min at room temperature, a confluent layer of red blood cells adhered to the monolayers except in the areas infected with rubella virus, which could be easily counted as clear plaques.

The sensitivity of this technique was tested by performing a series of 30 comparative titrations; simultaneously, each titration was carried out by the hemadsorption-negative plaque test and by the standard rubella assay method utilizing the inhibition of echovirus 11 cytopathic effect in GMK cells (15). The titrations in the latter instances were performed with four tubes per 10-fold dilution, and the IND_{50} was calculated by the method of Reed and Muench (19). The range of differences between the hemadsorption-negative plaque titers and the IND_{50} titers was +0.7 to −0.6 $\log_{10}$, with a mean difference of −0.12 $\log_{10}$ and a standard deviation of 0.28 $\log_{10}$.

Density gradient centrifugation. Rubella virus was banded in density gradients of sucrose, as previously described for vesicular stomatitis virus (12). In general, 0.5 ml of virus, concentrated 10- to 50-fold by pelleting in a Spinco #30 rotor at 30,000 rev/min for 1 hr, was layered over 4.5 ml of preformed gradient. Centrifugation at 36,000 rev/min (141,995 × *g*) in the SW-50 rotor was carried out in a Spinco model L2-65 ultracentrifuge. The gradients were fractionated by puncturing the bottom of the tube with a piercing unit (Buchler Instruments, Inc., Fort Lee, N.J.) and collecting either 20 fractions (20 drops each) or 40 fractions (10 drops each). The density curve for the gradient was computed by measurement of the refractive index of every second or third fraction in an Abbe 3L refractometer, and the density was extrapolated from standard curves obtained by use of data from the International Critical Tables. Periodically, the accuracy of this method was checked by direct weighing of samples in 100-μliter pipettes.

The density gradients used in this study were prepared as follows. (1) Sucrose (5 to 70%) was prepared in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 7.1) containing 0.001 M ethylenediaminetetraacetic acid (EDTA). The gradient was prepared by layering 0.4 ml of 70% and 0.3 ml of 5 to 65% in increments of 5%. (2) Sucrose (5 to 70%) was prepared in distilled water. The gradient was layered in the same fashion as in gradient 1. (3) Sucrose (nonlinear 5 to 60%) was prepared either in water or in the same Tris buffer containing EDTA (*p*H 7.1) as gradient 1. The gradient was prepared by layering 0.6 ml of 60, 55, and 50%, 1.4 ml of 45%, 0.6 ml of 40%, and 0.1 ml of 5 to 35% in 5% increments.

The gradients were held at 4 C for at least 2 hr to allow for diffusion of the layers.

RESULTS

The results of the recovery of rubella virus, obtained after concentration by ultracentrifugation at 30,000 rev/min (105,000 × *g*) for 1 hr and after density gradient centrifugation for 2 hr in sucrose, are given in Table 1. As can be seen, greater than 100% of the total virus in the crude tissue culture fluids was recovered after pelleting. This high recovery was consistently observed and may reflect the removal of some inhibitor or unmasking of infectious virus. The amount of rubella virus recovered after gradient centrifugation was also high, 93% in the experiment cited.

Rubella virus was banded in a preliminary experiment on a density gradient of Tris-buffered (*p*H 7.1) sucrose (5 to 70%) containing EDTA (gradient 1). In this experiment, the gradient was fractionated after 2 hr of centrifugation at 36,000 rev/min (Fig. 1), and each of the fractions was assayed for infectivity. The peak of infectivity corresponded to a density of 1.16 g/cm³. Since 76% of the infectivity was located in this peak, it was assumed that rubella virus had a density considerably higher than that reported by other workers. Similar results were obtained with virus strains R-1 and R-3, and no difference was observed with the R-1 virus strain grown in BHK_{21} or human embryonic lung cells.

However, when the virus was centrifuged in the same sucrose gradient for 18 hr, a somewhat higher density, about 1.18 g/cm³, was obtained (Fig. 2). Even though the difference between the densities of 1.16 g/cm³ and 1.18 g/cm³ in this particular gradient was only a matter of several fractions, the difference was consistent in all of the repeat gradients that were run. Despite the closeness of these two densities in the gradient, adequate separation was achieved, since, as seen in Fig. 1, a line drawn through fraction 15 (a

TABLE 1. *Recovery of rubella virus after concentration by ultracentrifugation or density gradient centrifugation*

Method of concn	Total PFU input	Total PFU recovered	Percentage of PFU recovered
Pelleting by ultracentrifugation[a]	3.0×10^6	4.5×10^6	150
Density gradient centrifugation[b]	4.5×10^6	4.2×10^6	93

[a] Centrifugation at 30,000 rev/min for 1 hr with the Spinco #30 rotor.

[b] Centrifugation at 36,000 rev/min for 2 hr in a sucrose gradient with the Spinco SW50 rotor.

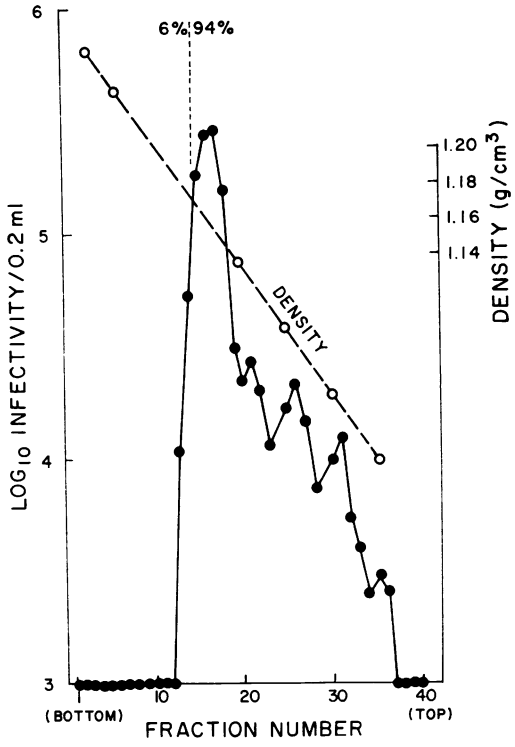

FIG. 1. *Distribution of rubella virus infectivity in fractions after 2 hr of centrifugation at 36,000 rev/min in a linear sucrose density gradient (5 to 70%) prepared in Tris-buffered EDTA. Symbols: ●, infectivity; ○, density.*

density of 1.17 g/cm³) reveals that only 6% of the total infectivity was found above this density, whereas in Fig. 2, 75% of the total infectivity was at a density greater than 1.17 g/cm³.

Evidence that the increase in density after the 18 hr of centrifugation was not simply a matter of the virus reaching equilibrium was obtained when rubella virus was centrifuged in a sucrose gradient (5 to 70%) prepared in distilled water (gradient 2). The density of peak of infectivity in the absence of the Tris-buffered EDTA was found to be 1.16 g/cm³, even after 18 hr of centrifugation, and the curve was similar to that illustrated in Fig. 1. Thus, it would appear that the presence of Tris-chloride buffer or EDTA or both in the sucrose gradient somehow caused rubella virus to assume a higher density.

The shift to the heavier density was found to be gradual in a kinetic study. In this study, rubella virus was placed on replicate Tris-buffered sucrose gradients containing EDTA, and the gradients were harvested after 2, 6, 8, and 21 hr of centrifugation (Fig. 3). A nonlinear gradient (gradient 3) was employed in order to achieve a better separation between the 1.16 and 1.18 g/cm³ density fractions. In this gradient, eight fractions separated these two densities, whereas, in the gradients used in Fig. 1 and 2, they were only separated by two fractions. The results clearly show that the peak of virus infectivity increased from a density of 1.157 g/cm³ at 2 hr to 1.167 g/cm³ at 6 hr, with densities of 1.175 g/cm³ and 1.185 g/cm³ after 8 and 21 hr of centrifugation, respectively.

The density shift was found to be the result of some effect of Tris-chloride buffer or EDTA or both on the virus; the shift was reversible (Table 2). In experiment A, a virus harvest was divided into two portions and pelleted by centrifugation. One pellet was resuspended in maintenance medium containing 2% fetal bovine serum, and the second pellet was resuspended in Tris buffer containing EDTA. After incubation for 18 hr at 4 C, the two virus suspensions were centrifuged on nonlinear sucrose gradients (gradient 3) for 2 hr. As seen in Table 2, preincubation with Tris-EDTA caused the virus to band at the higher density (1.18 g/cm³) after only 2 hr of centrifugation, whereas the parallel control resuspended in growth medium banded at 1.16 g/cm³. In ex-

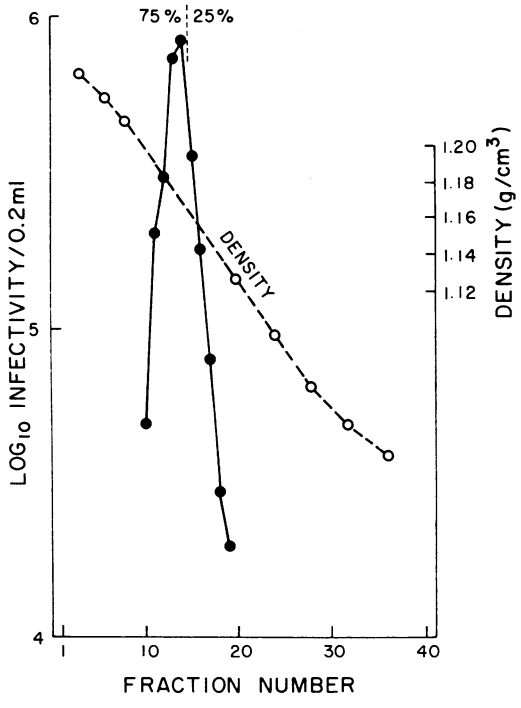

FIG. 2. *Distribution of rubella virus infectivity in fractions after 18 hr of centrifugation at 36,000 rev/min in a linear sucrose density gradient (5 to 70%) prepared in Tris-buffered EDTA. Symbols: ●, infectivity; ○, density.*

periment B, a virus harvest was concentrated by pelleting, and the resulting suspension was centrifuged on nonlinear sucrose gradients (gradient 3) for 2 and for 18 hr. After these periods of centrifugation, the virus at the 1.16 g/cm³ and 1.18 g/cm³ fractions was harvested and dialyzed against distilled water to remove both the sucrose and the Tris-EDTA. These two fractions were then rebanded for 2 hr on sucrose gradients. As can be seen, the virus obtained from the heavier 1.18 g/cm³ band after 18 hr of centrifugation was found on recentrifugation to band at the lighter density, as did the virus from the lighter 1.16 g/cm³ density. This series of experiments demonstrated that the density shift observed for rubella virus was dependent on the action of Tris-EDTA buffer and was reversible.

Antiserum to BHK_{21} cells was made with rabbits which had a cytotoxic titer of 1:256. This antiserum neutralized rubella virus grown in BHK_{21} cells at a final concentration of 1:20 but did not neutralize virus grown in human embryonic lung cells. Rubella virus prepared in BHK_{21} cells was obtained from the 1.16 g/cm³ and 1.18 g/cm³ fractions, and the virus from the fractions was neutralized equally well with the BHK_{21} antiserum. This suggested that the shift in density was not due to loss of host-cell components.

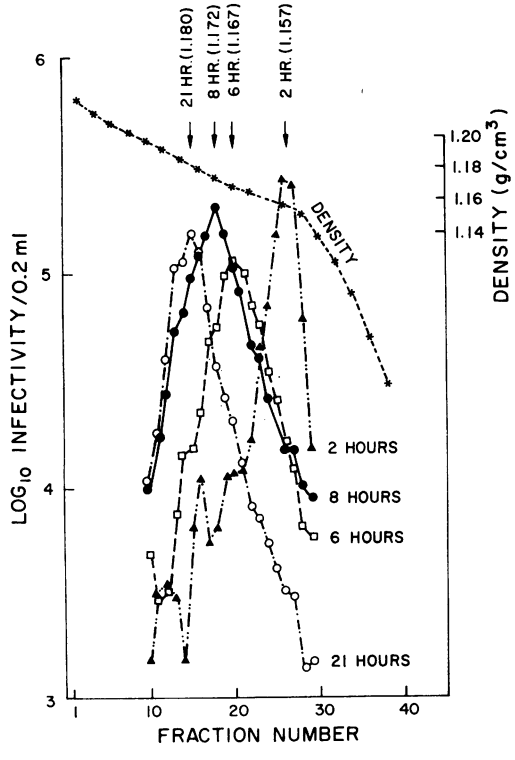

FIG. 3. *Distribution of rubella virus infectivity in fractions after 2, 6, 8, and 21 hr of centrifugation at 36,000 rev/min in replicate nonlinear sucrose density gradients. Symbols: ▲, infectivity after 2 hr; □, infectivity after 6 hr; ●, infectivity after 8 hr; ○, infectivity after 21 hr; ✳, density.*

TABLE 2. *Influence of Tris-buffered EDTA on the density of rubella virus*

Expt	Treatment of virus	Density peak of infectivity[a]
A	Virus pretreated for 18 hr at 4 C with:	
	Maintenance medium	1.16 g/cm³
	Tris buffer containing EDTA	1.18 g/cm³
B	Virus centrifuged in sucrose gradients containing Tris-buffered EDTA and rebanding of:	
	1.18 g/cm³ fraction collected after 18 hr	1.16 g/cm³
	1.16 g/cm³ fraction collected after 2 hr	1.16 g/cm³

[a] Centrifuged in a sucrose gradient for 2 hr at 36,000 rev/min.

DISCUSSION

The results of this study show that rubella virus can assume one of two densities, depending upon the state of the virus. Thus, virus centrifuged for 18 hr in sucrose gradients containing Tris-EDTA buffer, or virus pretreated with this buffer, had a density of 1.18 g/cm³. However, virus centrifuged in sucrose gradients prepared in distilled water, that is, in the absence of the Tris-EDTA buffer, had a density of 1.16 g/cm³, even after 18 hr of centrifugation.

The data indicate that the density observed for the virus after 2 hr of centrifugation in the Tris-EDTA-buffered sucrose gradient (Fig. 1) was a matter of not reaching equilibrium. This interpretation would adequately explain the results observed in Fig. 3, which shows rubella virus to be sedimenting with time toward the 1.18 g/cm³ region of the gradient. However, we showed that this was not the case, since virus centrifuged for 18 hr in sucrose gradients prepared in distilled water was found at a density of 1.16 g/cm³, as it also was after 2 hr of centrifugation. In addition, virus pretreated with the Tris-EDTA buffer banded at the heavier density (1.18 g/cm³) after only 2 hr of centrifugation (Table 2, experiment A).

The hypothesis that rubella virus lost some lipid-containing component in the presence of

TABLE 3. *Densities reported previously for rubella virus*

Viral activity measured	Gradient material	Density	Reference
		g/cm³	
Infectivity	Sucrose	1.12	14
Infectivity	Sucrose	1.07	6
Infectivity	Sucrose	1.075	2
Infectivity	Ficoll, CsCl	1.085	23
Complement fixation			
(a) Soluble	Sucrose	1.04	24
(b) Particulate		>1.149	
Complement fixation			25
(a) Soluble	Sucrose	1.08–1.14	
(b) Particulate (infective)		1.19–1.23	
Infectivity + hemagglutination	Sucrose	1.18	3
Infectivity	Potassium citrate	1.20	7

Tris-buffer and EDTA was made untenable by demonstrating the reversibility of the density shift (Table 2, experiment B). A lipid-containing cell component lost from the virus, resulting in the density shift from 1.16 g/cm³ to 1.18 g/cm³, would probably rise to the top of the gradient, and the chance of the reaggregation of the virus with this component would be very small. Also, since rubella virus can be neutralized by anti-host cell serum, the lack of difference in virus obtained from the two different densities further suggests that there was no significant loss of a contaminating host-cell component.

The most likely explanation for the observed density difference concerns the degree of hydration of the virus. It is possible that, in the presence of Tris buffer containing EDTA, the virus loses a certain amount of water and thus assumes a density of 1.18 g/cm³, whereas, after the removal of this buffer, the virus regains the lost water or rehydrates and has a density of 1.16 g/cm³. This explanation is consistent with all of the experimental evidence, but no direct evidence could be obtained to substantiate this hypothesis. Even though it was not possible to demonstrate a mechanism for the observed density shift, a hydration phenomenon that occurs under similar conditions has been described for subcellular fractions (11, 20). The phenomenon, termed water extrusion, has been observed in nuclei, mitochondria, and microsomal cellular fractions from rat liver and in spinach chloroplasts. It has been shown that these organelles extrude water in the presence of a divalent cation and EDTA. The reaction involves the phospholipids of these organelles. Since rubella virus matures by budding from host-cell membranes (R. M. McCombs, J. P. Brunschwig, and W. E. Rawls, Exptl. Mol. Biol., *in press*), and presumably obtains a lipid-containing membrane similar to that of the cell organelles, the possibility exists that the water extrusion phenomenon may be responsible for the density shift.

The density reported in this study was considerably higher than that reported by other workers (Table 3). Generally, those studies that reported a low density for this virus also reported low recoveries of infectivity. The low recoveries may have been due to the fact that the bottom of the gradient was not sufficiently dense and the majority of the virus sedimented onto the bottom of the centrifuge tube. This was confirmed with a sucrose gradient prepared as described by Cusumano (2). After centrifugation the gradient was fractionated, and between 50 and 70% of the infectivity initially added to the gradient was recovered as a pellet in the bottom of the centrifuge tube. This situation is somewhat analogous to the results obtained with the recently described satellite viruses, found to be present in many adenovirus preparations (1, 4, 10). In the purification of adenovirus in cesium chloride density gradients of 1.26 to 1.36 g/cm³ customarily used, the satellite virus was pelleted because of its higher density (1.38 to 1.43 g/cm³) and thus went undescribed for many years. However, it is also possible that the difference in the density of rubella virus that has been reported in the literature is due to other factors, such as the use of different cell lines or different methods of virus concentration.

More recently, reports have appeared that also show a higher density for rubella virus with potassium citrate (7) as well as sucrose (3, 23). The latter report is of particular relevance to our work, since a density of 1.18 g/cm³ was reported for the virus in a sucrose gradient containing EDTA.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-05382 and Research Career Development Award 1 K3 AI 25, 943 from the National Insti-

tute of Allergy and Infectious Diseases and by grant HE-05435 from the National Heart Institute.

The technical assistance of Pat Hill is appreciated.

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