Density Gradient Centrifugation Studies on Rabies Virus

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Abstract

NEURATH, A. ROBERT (The Wistar Institute, Philadelphia, Pa.), TADEUSZ J. WIKTOR, AND HILARY KOPROWSKI. Density gradient centrifugation studies on rabies virus. J. Bacteriol. 92:102-106. 1966.—Cesium chloride density gradient centrifugation of rabies virus revealed a heterogeneous population of infectious virus particles, the majority of which showed a density of 1.20 g/ml. From results obtained by rate zonal centrifugation in preformed sucrose gradients, it was possible to calculate a sedimentation coefficient of about 600S for rabies virus. Sedimentation coefficients of about 235 and 10S were calculated for two soluble rabies antigens present in infected tissue-culture fluids, and they showed a density of 1.26 g/ml in cesium chloride solutions.

Rabies virus has been characterized as a lipid containing ribonucleic (RNA) virus (4, 7, 8) which may belong to the same morphological group as vesicular stomatitis virus, coxalvirus, Egtved virus, and sigmavirus of Dro sophila (22). These correspond to the process sequence group 32S' of Kilkson (9). It has been suggested that the rabies virus may be a part of the myxovirus group (1).

Results contributing to the physical characterization of rabies virus, obtained by density gradient and rate zonal centrifugation techniques, are reported in this communication.

Materials and Methods

Rabies virus. The Pitman Moore (PM) strain of fixed rabies virus received from the National Institutes of Health (NIH, Bethesda, Md.) was used throughout the experiments. The virus was grown in human diploid cell strain (HDSC) for 52 passages and then in hamster kidney fibroblast culture BHK-21/C 13 for an additional three passages by a method described elsewhere (21). Infectious tissue-culture medium harvested on the 3rd day was used throughout the experiments. The titer of the virus was $3 \times 10^4$ LD$50/0.03$ ml in 5-week-old Swiss mice inoculated intracerebrally. In some experiments, the tissue-culture medium was concentrated (see below) before density gradient centrifugation.

Rabies virus antigen. The presence of rabies virus antigen was determined by a fluorometric method recently described (15). Fluorescein isothiocyanate conjugated antirabies γ-globulin obtained from BBL was used.

Cesium chloride equilibrium density gradient centrifugation. A 0.4-ml amount of virus suspension was layered over 4 ml of a CsCl solution with a density of about 1.22 g/ml adjusted to pH 7 by addition of 1.0 M phosphate buffer to a final concentration of 0.01 M. Centrifugation was performed in a Spinco SW 39 L rotor for 48 hr at 96,000 × g. After the bottom of the tube was punctured, samples were collected in test tubes. A fraction withdrawn from each sample was mixed with a 10-fold amount of 2% calf serum in phosphate-buffered saline and kept at −70°C until assayed for infectivity. The density of the samples was determined refractometrically (20).

Rate zonal centrifugation. A 0.4-ml amount of virus suspension was layered over 25 ml of a preformed sucrose gradient buffer at pH 7.0 with phosphate buffer to a final concentration of 0.01 M. It was then centrifuged in the Spinco SW 25.1 rotor. If the preparation of soluble antigen was centrifuged, 0.3 ml was layered over 4 ml of sucrose gradient solution and centrifuged in the Spinco SW 39 L rotor. Samples were withdrawn after centrifugation and titrated for infectivity and presence of soluble antigens. The concentration of sucrose in the samples was determined refractometrically, and the corresponding values of density and viscosity were found in the Handbook of Chemistry and Physics, 44th edition. The calculation of sedimentation coefficients ($S_{20,w}$) was calculated according to Martin and Ames (11), but without the use of an internal standard. The time of centrifugation was corrected for the time of acceleration and deceleration. Densities of 1.20 and 1.26 g/ml were used for the calculation of the values of sedimentation coefficients that corresponded to infectious virus and soluble antigens, respectively (see Results).
Protein determination. The concentration of protein in the samples obtained after density gradient centrifugation was determined by the method of Lowry et al. (10).

RESULTS

Concentration of virus by precipitation and centrifugation. Infectious tissue-culture fluids were concentrated by precipitation with zinc acetate, according to a method used for concentration and stabilization of respiratory syncytial virus (Spicer and Sweet, personal communication). In this procedure, 50 parts of tissue-culture fluid were mixed with 1 part of 1 M zinc acetate; and the suspension was kept for about 30 min at +4 C. The resulting precipitate was sedimented by low-speed centrifugation (2,000 rev/min in an IEC centrifuge, model PR-Z, head 284). The supernatant fluid was discarded and the sediment resuspended in a volume of buffer equal to one-twentieth of the original volume of tissue-culture fluid. The buffer was a saturated solution of disodium ethylenediaminetetraacetate adjusted by addition of solid tris(hydroxymethyl)aminomethane to pH 8. The suspension was dialyzed for several days at 4 C against a phosphate-buffered (0.01 M phosphate, pH 7.0) solution of NaCl (0.14 M; PBS) and then was titrated in mice. The results indicated a 20-fold increase in the concentration of infectious virus.

Infectious virus was separated from rabies virus “soluble” antigens and other tissue culture proteins by high-speed centrifugation either under conditions described elsewhere (2) or on a cushion of a CsCl solution. A 2-ml amount of this solution, buffered at pH 7.0 and with a density of 1.29 g/ml, was placed in a Luerdoid centrifuge tube for the Spinco SW 25.1 rotor. The tube contents were overlaid with 2 ml of a buffered CsCl solution of density 1.145 g/ml, and then with 20 ml of the virus concentrated by precipitation with zinc acetate. After 4 hr of centrifugation at 49,500 × g, four fractions were collected by puncturing the bottom of the tubes; the “cushion,” the interphase, the supernatant fluid, and a cloudy layer formed at the top of the tube. About 96% of rabies virus infectivity determined by mouse inoculation was recovered in the interphase, and 3.5% was recovered in the supernatant fluid. By contrast, 90% of rabies antigen bound by the fluorescent antibody (see Materials and Methods) was recovered in the supernatant fluid.

Analysis of fractions obtained after CsCl density-gradient centrifugation. The results of CsCl density gradient centrifugation of rabies virus are presented in Fig. 1B. Infectious virus was recovered from a relatively large number of fractions, which suggests a heterogeneity of the virus population with respect to density. The majority of particles had a density of 1.20 g/ml. Fractions no. 7 and 10 were then centrifuged separately in CsCl density gradients, under the same conditions as was the original material, to exclude the possibility that the broad peak of infectivity could have been an artifact due to experimental conditions. Results shown in Fig. 1A suggest that heterogeneity seems to be an intrinsic property of the virus population.

When the supernatant fraction, obtained after centrifugation on CsCl cushion (see above), was subjected to density gradient centrifugation, the band (Fig. 2) containing the bulk of soluble antigens had a density of 1.26 g/ml, i.e., higher than that of the infectious virus.

Analysis of fractions obtained after rate-zonal centrifugation in sucrose gradients. In two separate experiments with nonconcentrated infectious tissue-culture medium, fraction 10 contained the

![Fig. 1](http://jb.asm.org/)

*Fig. 1. CsCl equilibrium density-gradient centrifugation of rabies virus. Fraction 1 = bottom; fraction 15 = top. (A) Recently fractionated fractions no. 7 (○) and no. 10 (▲) from part B (centrifugation of infectious tissue-culture fluid). Symbols: □ = density; ○, ▲ = infectivity.*
greatest amount of infectious virus: 60% (Fig. 3) and 37%, respectively. This position in the gradient corresponds to a sedimentation coefficient of approximately 600S.

Figure 4 shows results obtained after centrifugation of a virus suspension concentrated 3,000 times by precipitation with zinc acetate and high-speed centrifugation. This suspension contained $10^{2.4} \text{LD}_{50}/0.03 \text{ml}$. The values for sedimentation coefficients calculated from these data were similar to those obtained with the crude suspension, and a good correlation between the distribution of infectivity and concentration of antigen was found.

Results of centrifugation in sucrose of a preparation of soluble antigens (Fig. 5) indicate the presence of two peaks of antigenic activity for which sedimentation coefficients of 23S and 10S were calculated.
DISCUSSION

It has been shown that preparations of rabies virus consist of particles differing in buoyant density—a phenomenon previously described for other mammalian viruses. It occurs in mutants of encephalomyocarditis virus (5) and different plaque types of herpes simplex virus (18). In addition, Newcastle disease virus particles produced in a single cell type (19), Rous sarcoma (3), and lymphocytic choriomeningitis LCM virus particles (16) are heterogeneous with respect to density. Numazaki and Karzon (Bacteriol. Proc., p. 110, 1965) found two different density types with intracellular measles virus and suggested that one type might be the precursor of the other.

In the case of rabies virus, it remains to be determined whether the density heterogeneity revealed by centrifugation in cesium chloride is due to an association of virus particles with host components and whether the difference in densities has a genetic basis.

Rate zonal centrifugation in sucrose gradients revealed that rabies virus has a sedimentation coefficient of about 600S. The occurrence of a small fraction of infectious virus at positions in the gradient corresponding to much lower values of sedimentation coefficient needs further investigation. Results that suggest a heterogeneity of virus particles with respect to sedimentation velocity have also been obtained with I CMV (16).

Very recently, McCombs et al. (12) reported results for vesicular stomatitis virus that are similar to our findings for rabies virus with respect to density and heterogeneity.

The shoulder peak of infectivity after rate zonal centrifugation under the conditions given in Fig. 4 probably reflected separation of particles of different size. Aggregation of virus particles may explain the shoulder to the infectivity peak and the two peaks of antigen (Fig. 4).

Rate zonal centrifugation of a preparation of soluble antigen revealed two main components with sedimentation coefficients of about 23S and 10S. This result is compatible with the demonstration of two precipitin lines by the Ouchterlony technique (6). Although minor peaks of antigenic activity were detected in both sucrose and CsCl density gradient centrifugation experiments, it is not yet certain whether they represent background noise or are specific antigens. The latter possibility should be considered, since more than two precipitating antigens seemed to be present in virus-free extracts of infected suckling mouse brains (14). The relationship between the complement-fixing antigens, found in infected mouse brains (13, 14), and the precipitating antigens should be studied to determine whether the 23S particle is identical with the previously described soluble complement-fixing antigen of 12 μm diameter (17). It also remains to be determined whether or not the two soluble antigens described have the same antigenic determinants, and what their relationship is to the complete virion.

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