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Purification of Rabies Virus Grown in Tissue Culture

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Extracellular rabies virus, grown in monolayer cultures of BHK21 cells in the presence of medium supplemented with bovine serum albumin, was purified by the following procedure. Virus was precipitated from infectious tissue culture fluid by zinc acetate and was resuspended in a solution of ethylenediaminetetraacetate. The suspension was filtered through a Sephadex column and was treated with ribonuclease and deoxyribonuclease. The virions were then pelleted by centrifugation at high speed and were resuspended in buffer solution. Banding of the virus by centrifugation in a sucrose density gradient was the final step in the purification procedure. Purified preparations contained bullet-shaped virus particles of variable length and little (up to 5%) contaminating host-cell material. Most of the virions were "complete", i.e., 180 nm long, but some virus particles were shorter. The length distribution of the virions was nonrandom. Shorter virions seemed to be noninfectious and showed markedly decreased hemagglutinating activity. The complement-fixing activity and the ribonucleic acid to protein ratio of the virions were not related to the length of the virus particles. Although the properties of extracellular and intracellular viruses were similar, the procedure was not suitable for purification of intracellular rabies virus.

Large quantities of highly purified virus are necessary for the study of the antigenic structure and the chemical composition of the virion. Modified methods for cultivating rabies virus in tissue culture, described in the present report, have made it possible to obtain increased yields of virus. Similar improvement in methods for quantitative assay of the virus (24) had previously opened the way to purification of rabies virus grown in tissue culture. This report summarizes the results of experiments in which an efficient recovery rate of highly purified virus was achieved. Some properties of purified rabies virons are also described.

MATERIALS AND METHODS

Virus strain. The Pitman-Moore (PM) and Flury high egg passage (HEP) strains of rabies virus, adapted to growth in human diploid cell strain WI-38 and propagated in these cells for 52 and 16 passages, respectivelv (30), were three times clone-purified by plaquing in agarose-suspended BHK/13S cells (24). Seed pools of virus were prepared in BHK21 cell cultures.

Tissue cultures. Two hamster cell lines, BHK21 clone 13 (17) and Nil-2 cells (4), were propagated in 1-liter Blake bottles as described previously (14).

Buffer solutions used in virus purification. NT buffer was composed of 0.13 M NaCl and 0.05 M tris(hydroxymethyl)aminomethane (Tris) - chloride (*p*H 7.8); NTE buffer was composed of 0.13 M NaCl, 0.001 M ethylenediaminetetraacetate (EDTA), and 0.05 M Tris-chloride (*p*H 7.8).

Infection of cells and virus propagation. After removal of the growth medium, cell cultures were infected with 2 ml of seed virus containing 50 μg of diethylaminoethyl (DEAE) dextran per ml (Pharmacia Fine Chemicals, Piscataway, N.J.). DEAE dextran was added to increase the efficiency of virus adsorption and penetration (14). After 1 hr of adsorption at 35 C, the inoculum was removed; the cell monolayers were washed once with warm phosphate-buffered saline (6), and were refed with growth medium containing either 10% heat-inactivated calf serum or 0.4% bovine serum albumin (BSA; fraction V; Nutritional Biochemicals Corp., Cleveland, Ohio) and a doubled amount (0.44%) of sodium bicarbonate. Cultures were then incubated for 60 to 72 hr at 32 to 33 C. The input multiplicity of infection was between 0.5 and 2 plaque-forming units (PFU) of virus per cell. No specific changes could be detected in the infected cells when cultures were examined with the light microscope at the time of harvest.

Assay of mycoplasma. The infected and noninfected cell cultures used in the present study were tested for the possible presence of mycoplasma by a method described previously (31). No cultures were contaminated.

Titration of virus infectivity. Virus was assayed by a plaque technique in agarose-suspended BHK/13S cells. The original method (24) was modified as

follows. Petri dishes containing one layer of nutrient agarose and a superimposed layer of agarose-cell mixture (31) were incubated for 24 hr and then infected with 0.1 ml of serial virus dilutions. The virus inoculum was placed on the surface of the cell-containing agarose layer and was allowed to penetrate. Infected plates were incubated for 7 days at 35 C in a humidified CO_2 incubator before the addition of neutral red. Plaques were counted 4 hr later. In some experiments, the PM virus was also titrated by intracerebral inoculation of 4- to 5-week-old white Swiss mice. One MLD_{50} was found to correspond to approximately 10 PFU of this virus strain.

Assay of viral hemagglutinin (HA). The method devised recently by Halonen et al. (8*a*) was used. Serial twofold dilutions of virus preparation were made in a solution of 0.12 M NaCl and 0.05 M borate (*p*H 9.0), containing 0.4%, BSA, and the dilutions were kept at 4 C for 30 min. An equal volume of 0.25% goose erythrocytes suspended in 0.15 M NaCl-0.2 M phosphate solution (*p*H 6.4) was then added to each well of a plastic plate. The end point of the reaction was read after incubation for 45 min at 4 C. The reciprocal of the highest final dilution showing partial hemagglutination was considered to represent the number of hemagglutination units (HAU) per milliliter. When titers were so expressed, the results of titrations carried out in plastic plates (0.1 ml final volume) and in test tubes (1 ml final volume) were identical.

Complement fixation (CF) test. The microtechnique described by Sever (25) was used. The volume of each ingredient used was 0.05 ml. The amount of antirabies immune serum used for titration of the antigen corresponded to 10 antibody units, as determined on the basis of 50% hemolysis in tube tests. This amount of serum did not show a prozone phenomenon in block titration. The reciprocal of the highest initial dilution of antigen active in the test was considered as the number of complement-fixing units (CFU) per ml. This way of expressing the titers gave identical results for titrations carried out with plastic plates (0.25 ml final volume) and for titrations carried out with test tubes (1 ml final volume).

Immune sera. Antirabies immune sera, obtained from rabbits immunized with concentrated and partially purified viral antigen (strain PM), was depleted of host cell antibodies by absorption with Nil-2 cells. A serum dilution of 1:60,000 (0.05 ml) reduced by 50% the number of plaques formed by 40 PFU of rabies virus. When diluted as much as 1:1,200, the serum reacted in the CF test with 10 antigen units of rabies virus. It did not react with hamster cell antigens or BSA.

Anti-BHK-cell serum, obtained from rabbits immunized with disrupted cells, reacted in the CF test with 10 units of homologous antigen when diluted as much as 1:320.

The anti-BSA serum was purchased from Nutritional Biochemicals Corp.

Agar gel precipitation. The precipitin test was performed in 1% agarose gel. A 0.2-ml amount of immune serum was placed in the center well, and 0.1-ml amounts of antigen in the peripheral wells. The distance between the central and peripheral wells was 1 cm. The plates were read after incubation in a wet chamber for 7 days at 37 C.

Protein determination. Protein was determined according to the method of Lowry et al. (16), with BSA as the standard.

Determination of radioactivity. Acid-insoluble radioactivity was determined in a Beckman liquid scintillation counter by the usual techniques.

Electron microscopy. The preparations were examined by the negative-contrast technique. Suspensions of virus were transferred to carbon-coated Formvar grids by means of a platinum loop. A 4% solution of phosphotungstic acid (*p*H 6.8) was added, and the excess fluid was removed from the grid with filter paper. The specimens were photographed at a magnification of 10,5000 × or 53,000 × in a Siemens Elmiskop I electron microscope.

Preparation of radioactive virus. To prepare rabies virions containing labeled ribonucleic acid (RNA), the medium added to virus-infected cell cultures after virus adsorption was supplemented with 1.5 μc of ^{3}H-uridine per ml (16 c/mmole: Nuclear-Chicago Corp., Des Plaines, Ill.). The labeled precursor remained in the culture during the whole period of virus propagation.

Precipitation of the virus by zinc acetate. The procedure of D. Spicer and B. H. Sweet (*personal communication*; *see also* 20) was used with slight modification. Fifty parts of infectious tissue culture fluid, freed of cell debris by centrifugation at 1,000 × *g* for 20 min, was mixed with one part of 1 M zinc acetate solution (*p*H 5.0). After mixing, the *p*H of the virus suspension dropped from 7.2 to 7.4 to 6.7 to 6.9. The mixture was allowed to stand at 4 C for 20 min and then was centrifuged at 1,000 × *g* for 40 min; the supernatant fluid was discarded. The pellet was suspended in a saturated solution of disodium EDTA (about 11.7 g/100 ml of water), adjusted to *p*H 7.8 by the addition of solid Tris. The volume of the EDTA solution used for suspending the virus was 1.25% of that of the infectious tissue culture fluid. The resulting solution was clarified by centrifugation at 1,000 × *g* for 20 min.

Filtration through Sephadex column. Columns (33 × 3 cm) of Sephadex G-75 (particle size 40 to 120 μ; Pharmacia Fine Chemicals) were equilibrated with NT buffer. Crude virus suspension (10 ml) concentrated by precipitation with zinc acetate was then put on the top of the column, and the virus was eluted by washing with NT buffer at a flow rate of 1 ml per min.

High-speed centrifugation. The virus was pelleted by centrifugation at 49,000 × *g* at 4 C for 60 min. The supernatant fluid was discarded and the pellet was suspended in 0.5 to 0.7 ml of NTE buffer. With virus grown in the presence of serum, sonic treatment for 30 to 60 sec at 10 kc (Raytheon, model DF 101 sonic oscillator) was necessary to disperse the pellet. The resulting suspensions were then clarified by centrifugation at 1,000 × *g* for 20 min.

Sucrose density gradient centrifugations. About 0.5 ml of sample was layered on 28 ml of a linear gradient of sucrose in NTE buffer and was centifuged at 4 C in the SW 25.1 rotor of a Spinco centrifuge. When 10 to 60% (w/v) sucrose gradients were used, the

sample was centrifuged for 90 min at 61,000 × *g*. With 5 to 25% (w/v) gradients, centrifugation at 49,000 × *g* for 20 min was used. Fractions for analysis were collected through a hole punctured in the bottom of the tube.

Sedimentation of the virus on a cushion of sucrose solution. A 5-ml amount of 20% (w/v) sucrose solution in NTE buffer was placed on top of the same volume of 60% sucrose. About 19 ml of virus suspension, concentrated by precipitation with zinc acetate and dialyzed exhaustively against NT buffer or filtered through a Sephadex column, was layered on top of the 20% sucrose solution. The contents of the tube were then centrifuged in the SW 25.1 rotor of a Spinco centrifuge at 54,000 × *g* at 4 C for 3.5 hr. The virus band was collected by puncturing the bottom of the tube and was dialyzed exhaustively against NT buffer.

Release and concentration of cell-associated virus. Cell monolayers labeled with ^{3}H-uridine (1.5 μc per ml of medium) between 1 and 72 hr after infection were washed three times with NT buffer. The cells were scraped off and pelleted by low-speed centrifugation. The pellet was suspended in 0.5 M EDTA-Tris (*p*H 7.8), and the cells were disrupted by three cycles of freezing and thawing, followed by sonic treatment for 3 min at 10 kc. The suspension was centrifuged at 1,000 × *g* for 20 min, and the sediment was discarded. The released virus was pelleted by centrifugation at 49,000 × *g* at 4 C for 60 min. The pellet was suspended in 0.5 ml of NTE buffer by sonic treatment for 3 min, and the suspension was clarified by centrifugation at 1,000 × *g* for 40 min. The supernatant fluid was fractionated by sucrose density gradient centrifugation.

In control experiments with mock-infected cells, the same procedure was used, except that the cultures were labeled with ^{14}C-uridine (0.05 μc per ml of medium; 50 mc/mmole; New England Nuclear Corp., Boston, Mass.).

RESULTS

Attempts to purify extracellular rabies virus grown in the presence of serum. Our first attempts at purification were done with virus grown in the presence of serum. The virus was concentrated by precipitation from the infectious tissue culture fluid with zinc acetate. All of the infectivity was recovered when the precipitate was resuspended in EDTA solution, providing that the concentration of zinc acetate was adjusted to 0.01 to 0.02 M. At the routinely used 0.02 M concentration (i.e., when 100 parts of infectious tissue culture fluid was mixed with 2 parts of 1 M zinc acetate solution), about 25% of the serum proteins coprecipitated with the virus so that only a three- to fourfold increase in purity was achieved (Fig. 1). The impurities of low molecular weight were removed from the suspension without loss in infectivity either by dialysis against the NT buffer or by filtration through a Sephadex column. When

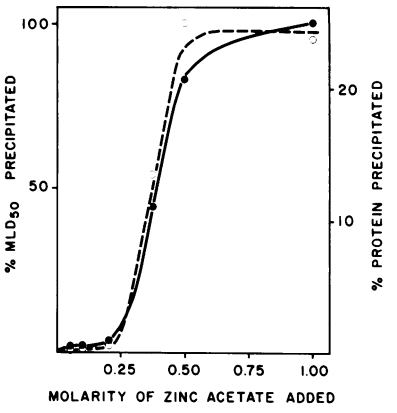

FIG. 1. *Efficiency of precipitation of rabies virus (strain PM) by various amounts of zinc acetate. Samples (30 ml) of infectious tissue culture fluid were treated with 0.6 ml of zinc acetate solution at various concentrations. The precipitate was dissolved in EDTA solution and assayed as described in Materials and Methods. Symbols:* ○, *recovery rate of infectivity;* ●, *recovery rate of proteins. The virus was grown in Nil-2 cells in the presence of medium supplemented with 10% calf serum.*

the virus was subsequently sedimented, the pellet could not be completely dispersed in the NTE buffer either by sonic treatment or by homogenization. An average of 80% of the virus was removed from the suspension in the form of large aggregates by low-speed centrifugation. Therefore, instead of pelleting the virus, it was sedimented onto a cushion of 60% sucrose solution. About 95% of the serum proteins present in the concentrated preparation were removed at this step, essentially without loss of infectivity. After the removal of sucrose by dialysis, however, a twofold decrease in the infectivity of the preparations was regularly observed. The virus was then pelleted, resuspended in NTE buffer by sonic treatment, treated with ribonuclease and deoxyribonuclease, and banded by centrifugation in a sucrose density gradient (Fig. 2A). Only 10 to 20% of the infectious units contained in infective tissue culture fluid were recovered in the viral band. The final preparation still contained 30 to 40% of the host cell and serum (e.g., γ-globulin) components, as shown by CF tests and by "purification" of mock-infected tissue culture fluid (Fig. 2B). In the electron microscope, the virus appeared in large aggregates and with pronounced distortion of its morphology. It was not possible to see the fine structure of the virus particles.

Purification of extracellular virus grown in the presence of BSA. To avoid difficulties caused by binding of serum substances to the virions, serum

was replaced by BSA in media used for virus propagation. The absence of serum did not reduce the virus yield. Infectious tissue culture fluids harvested 60 to 72 hr after infection contained 2×10^7 to 10×10^7 PFU of virus per ml. The final procedure, elaborated and successfully used for purification of extracellular rabies virus, is shown schematically in Fig. 3.

As with virus grown in the presence of serum, the virus grown in the presence of BSA was quantitatively precipitated in 0.02 M zinc acetate. However, only 2 to 5% of the proteins contained in the infectious tissue culture fluid were coprecipitated with the virus. Consequently, an appreciable increase in purity was achieved (Table 1). The purpose of filtration of the concentrated virus through Sephadex (Fig. 4) was to remove all of the low molecular weight impurities, rather than BSA. The presence of BSA at this and at the next two stages of the procedure was required for the stabilization of the biological activity of the virus. Treatment with ribonuclease and deoxyribonuclease followed by pelleting of the virus by high-speed centrifugation reduced the contamination of the preparation by nonviral nucleic acids

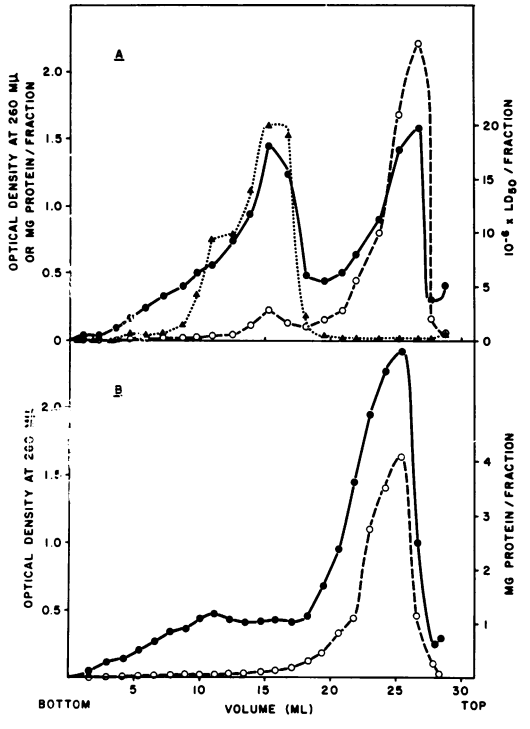

FIG. 2. *Sucrose density gradient centrifugation of concentrated and partially purified preparations of extracellular rabies virus (strain PM) grown in the presence of serum. (A) The virus was grown in Nil-2 cell cultures supplemented with medium containing 10% calf serum. It was precipitated by zinc acetate, and the precipitate was dissolved in EDTA solution. The virus was then sedimented on a cushion of 60% sucrose solution. The sucrose was removed from the virus suspension by dialysis, and the virus was pelleted. The resuspended pellet was layered on a 10 to 55% (w/w) linear sucrose gradient. Symbols: ○, protein content; ●, optical density at 260 nm; ▲, infectivity. (B) Control experiment with corresponding amount of noninfectious tissue culture fluid processed in the same way. Symbols: ○, protein content; ●, optical density at 260 nm.*

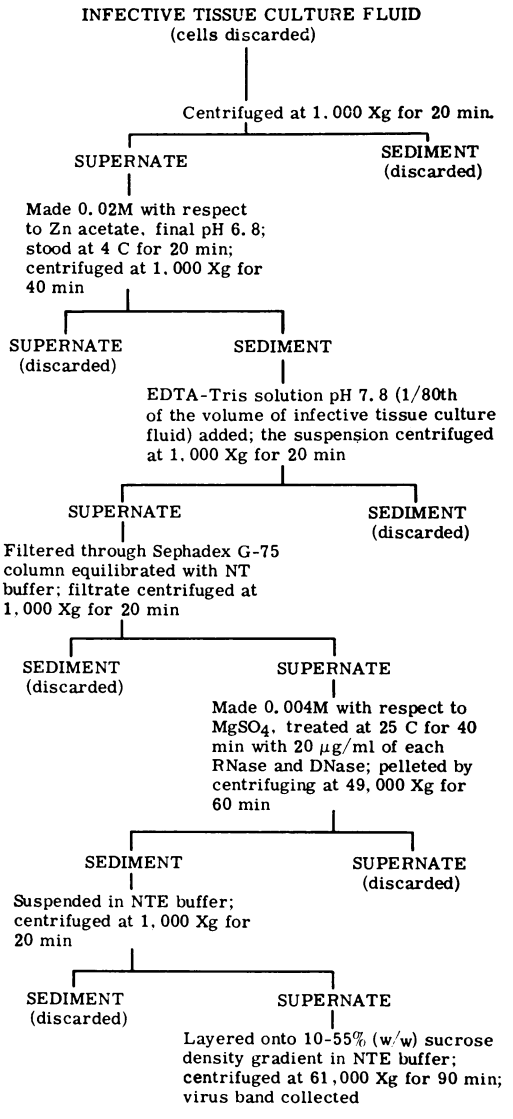

FIG. 3. *Schematic representation of the procedure used for the purification of extracellular rabies virus grown in cell culture in medium containing BSA in place of serum.*

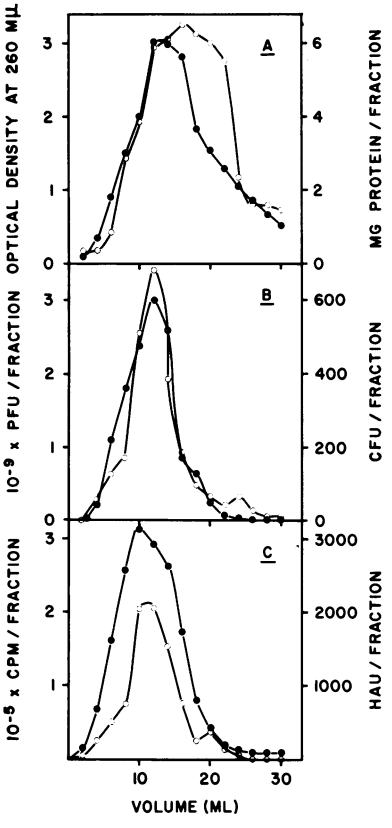

FIG. 4. *Filtration, through a Sephadex G-75 column, of crude rabies virus preparation (strain HEP) concentrated by precipitation with zinc acetate. (A) Symbols: ○, protein content; ●, optical density at 260 nm. (B) Symbols: ○, CFU; ●, PFU. (C) Symbols: ○, HAU; ●, radioactivity of ^{3}H-uridine. The three parts of the figure represent the same filtration experiment. The virus was grown in BHK cells in the presence of BSA.*

and eliminated most of the BSA. The transparent, bluish-opalescent pellet dissolved instantly in buffer with gentle shaking. No clumping or aggregation of the virus particles was observed when the suspension was examined under the electron microscope (Fig. 5). At this stage, the preparation was already of high purity (Table 1), but it still contained some BSA and host cell components. BSA could be removed easily with no loss of infectivity by repeated pelleting of the virus, since highly concentrated virus preparations were relatively stable even in the absence of BSA. Density gradient centrifugation had to be used, however, for the removal of cellular materials. Most of the impurities sedimented faster or slower than the virus (Fig. 6), so that they could be separated from the virions. Similar results were obtained when the virus was banded in a 5 to 22% (w/w) sucrose density gradient, except that the band was somewhat broader.

The peak of turbidity in the visible band (Fig. 6 and 7A) coincided with the peaks of protein content, radioactivity of incorporated RNA precursor, HA and CF activities, as well as infectivity of the virus. This coincidence indicated that the population of virions was homogenous with respect to various viral activities, when analyzed by rate zonal centrifugation in sucrose density gradient. As will be shown later, however, this homogeneity was only seen when the virus was derived from cultures exhibiting no sign of cell degeneration at the time of harvest. Light, noninfective HA or CF antigens were not detected in virus preparations processed by the method outlined.

Data characterizing the course of the purification at different stages of the procedure are summarized in Table 1. It is evident that the precipitation by zinc acetate and the digestion by enzymes following pelleting of the virus were the most efficient steps. Up to the banding of the virus in the sucrose density gradient, all of the original infectivity was usually recovered. After the banding, however, half of the original infectivity was usually lost. In spite of this, banding in a sucrose density gradient is unavoidable, if products essentially free of cell material are required. The final preparations were 1,000 to 3,000 times purer than the infectious tissue culture fluid, as judged by the ratio of infectivity to protein content.

Populations of extracellular virus exhibiting heterogeneity with respect to various viral activities. As was mentioned before, virus preparations obtained from cultures showing no gross signs of cell degeneration at the time of harvest were homogeneous. All viral activities sedimented together in the sucrose density gradient (Fig. 6). However, when 3-day-old confluent monolayer cultures, showing markedly decreased *p*H of the medium (6.8 to 7.1) and already containing some degenerated cells, were infected and incubated for 3 additional days, a large number of cells became detached from the glass. This effect was not due to the virus infection, since a similar degree of cell degeneration was observed in uninfected control cultures. The infectivity of the fluid harvested from such cultures was similar to, or even slightly higher (5×10^7 to 50×10^7 PFU/ml) than, that of fluids obtained from cultures showing no signs of cell degeneration. After removal of cell debris, extracellular virus was purified by the usual procedure. The final sedimentation revealed that the virions were heterogeneous with respect to their biological activity (Fig. 8, Table 2). The heavier virus particles had high infectivity and HA activity, whereas the lighter virus particles had little, if any, infectivity and decreased HA activity. The CF activity and the RNA content per milligram of

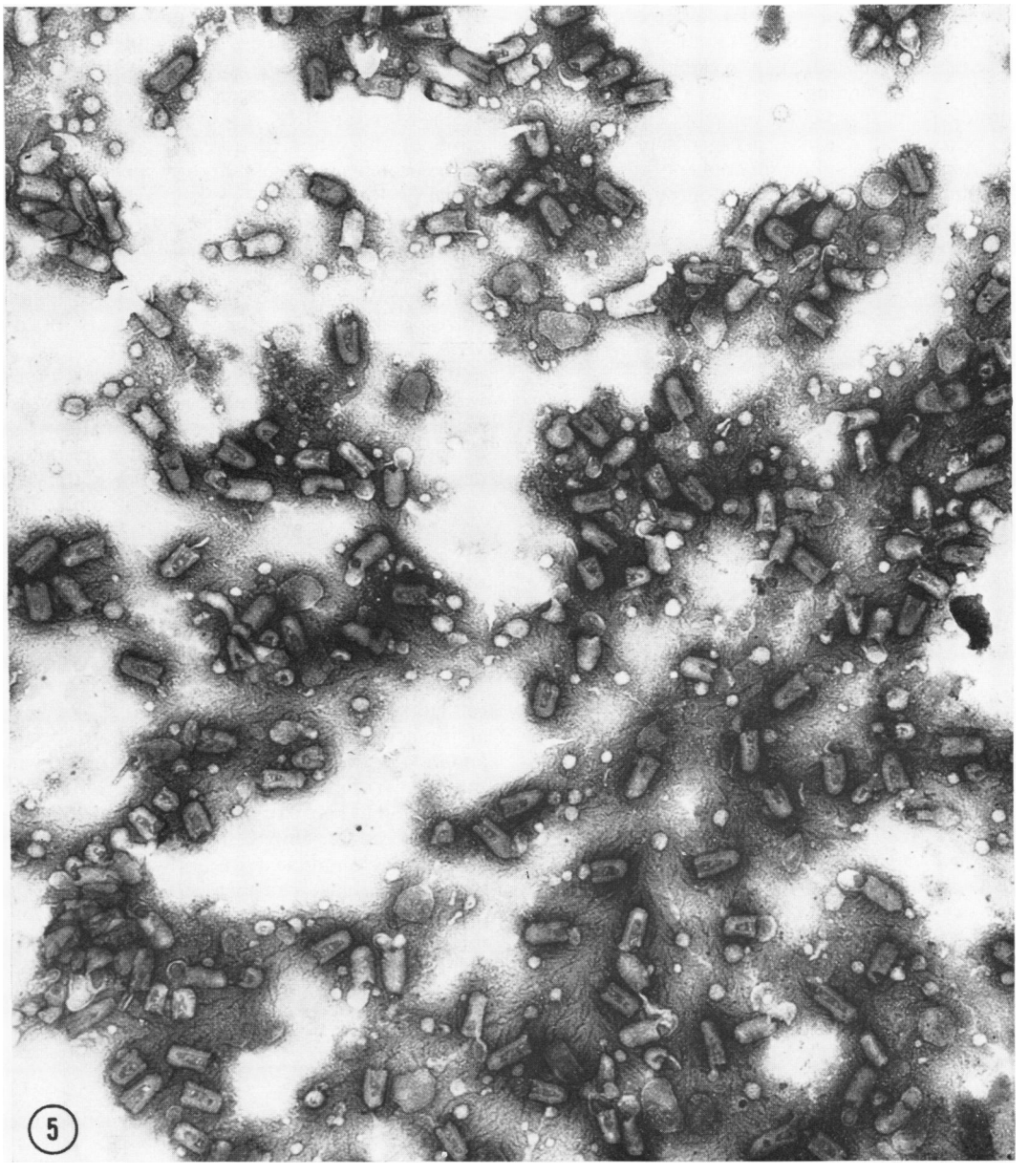

FIG. 5. *Electron micrograph of partially purified, extracellular rabies virus (strain HEP). The virus was grown in BHK cells in the presence of BSA. It was purified by the usual procedure, but banding in sucrose density gradient was omitted. Almost all PFU contained in the infective tissue culture fluid were recovered in such preparations. The particles are recognizable by their characteristic shape and their clumping is minimized. The preparation still contained host cell components and BSA.* ×*37,800.*

the heavy and light particles were similar (Table 2). Attempts to separate more efficiently the noninfective virus particles from infectious virus in a less steep (5 to 22% w/w sucrose) gradient were not successful.

Properties of purified virus preparations. The specific activities of purified rabies virus, which was shown to be homogeneous by sucrose density gradient centrifugation, were as follows: PFU per ml of protein = 10^{10}; HAU per mg of protein = 10^{4}; CFU per mg of protein = 5×10^{3}.

The buoyant density of the purified virus in

sucrose was found to be 1.17 after centifugation at 61,000 × *g* for 18 hr. This finding indicated that the final step usually used in the purification procedure, i.e., banding in a 10 to 55% (w/w) linear gradient of sucrose by centrifugation at 61,000 × *g* for 90 min, represents essentially a velocity rather than an equilibrium gradient centrifugation. Under the conditions used for purification, the virus banded at 1.14 density.

Various approaches were used to estimate the degree of contamination of purified virus preparations by host cell components and by BSA. Serological data summarized in Table 3 show that the purified virus did not react with anti-BSA serum. The virus did react, however, to a small extent with anti-BHK serum. Quantitative evaluation of the cross-reaction indicated that 10% of the proteins in the preparation were of cellular origin.

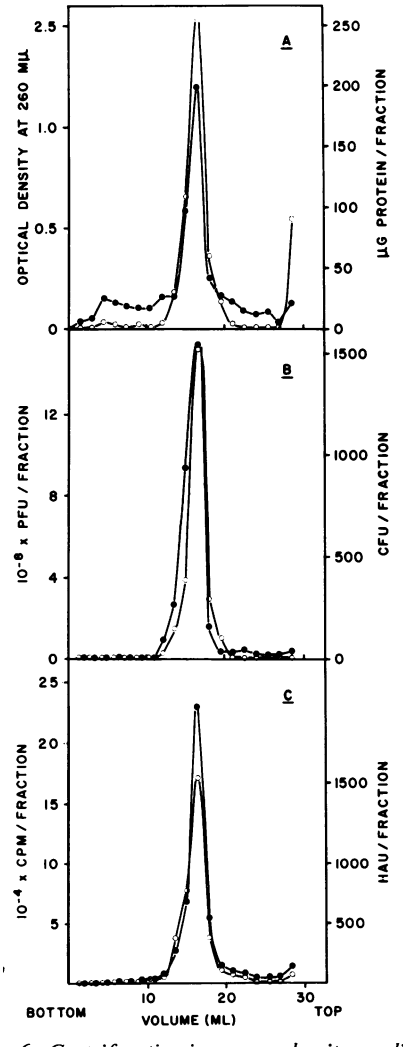

FIG. 6. *Centrifugation in sucrose density gradient of concentrated and partially purified rabies virus (strain HEP). A linear gradient of sucrose (10 to 55%, w/w) was used. (A) Symbols: ○, protein content; ●, optical density at 260 nm. (B): ○, CFU; ●, PFU. (C) Symbols: ○, HAU; ●, radioactivity of ^{3}H-uridine. The three parts of the figure represent the same centrifugation experiment. The virus was grown in BHK cells in the presence of BSA.*

Reaction of purified virus with specific antirabies serum in agar gel usually yielded a single precipitation line located very close to the antigen well. Occasionally, two additional lines, probably corresponding to breakdown products of the virus, were seen (Fig. 9).

When fluid from mock-infected tissue cultures was processed as outlined in Fig. 3, little host material was recovered from the final sucrose density gradient. From the recovery of optical density units (260 nm) at the position of the virus band from comparable amounts of noninfectious and infectious tissue culture fluids, it was estimated that purified virus preparations contain, at the most, 4% impurities.

Additional evidence that the contamination of the purified virus preparation by host material was small came from the following experiments. Noninfectious tissue culture fluid, derived from cell cultures labeled for 66 hr with 0.25 μc of ^{3}H-amino acid mixture (protein hydrolysate) per ml and 0.06 μc of ^{14}C-uridine per ml, was mixed with the same volume of unlabeled infectious tissue culture fluid. The virus was then purified by the usual procedure. Only 0.1% of the ^{3}H- and ^{14}C-acid-insoluble radioactivities originally present in the crude virus suspensions was recovered in the purified preparation. On the other hand, when infective tissue culture fluid labeled under similar conditions was mixed with an equal volume of unlabeled noninfectious tissue culture fluid and the virus was purified, 4% of the ^{3}H- and 5% of the ^{14}C-acid-insoluble radioactivity were recovered in the final preparation.

Electron microscopic investigations revealed that virus particles retained their structure after being precipitated with zinc acetate, filtered through Sephadex, and pelleted (Fig. 5). Virions were recognized in such preparations by their characteristic shape and architecture. Clumping of the virions was minimized, although the preparation still contained host cell material and BSA. Subsequent banding in a sucrose gradient eliminated most of the nonviral material. The increased purity of the preparation was also clearly demonstrable by electron microscopy (Fig. 10). Exposure of the virus to sucrose solution of high concentration, however, and the subsequent dialysis to remove sucrose (i.e., changes in the osmotic pressure) resulted in partial or total breakdown of many virions, reflected in a fivefold average reduc-

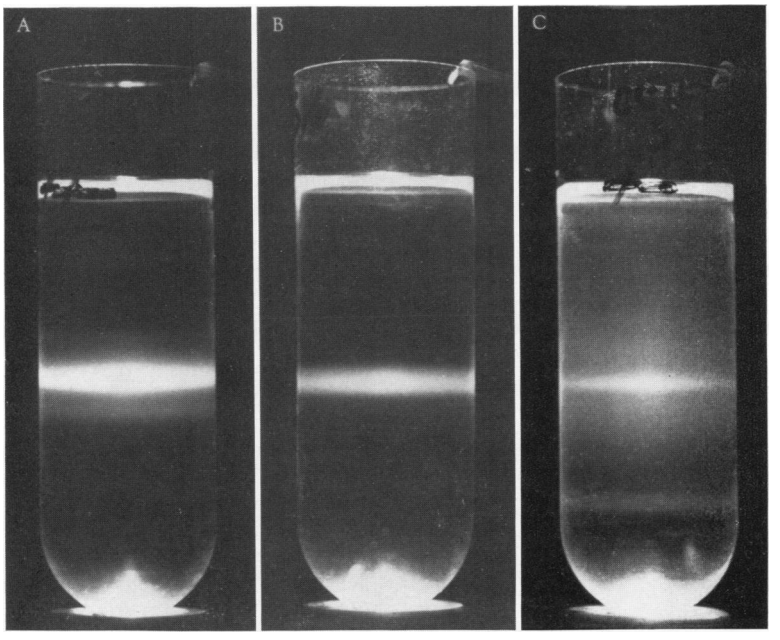

FIG. 7. *Banding of extracellular and cell-associated rabies virus (strain HEP) in a sucrose density gradient. (A) Extracellular virus, first banding. (B) Extracellular virus, rebanded. (C) Cell-associated virus, first banding; The virus band is located in the middle of the tube, whereas the band close to the bottom of the tube corresponds to cellular material. A linear gradient of sucrose (10 to 55%, w/w) was used. The virus was grown in BHK cells in the presence of BSA.*

TABLE 1. *Purification of the HEP strain of rabies virus*[a]

Sample	Vol	Amt of protein per ml	PFU/ml	HAU/ml	CFU/ml	Counts per min per ml[b]	PFU per mg of protein	Recovery rate (%)			
								PFU	HAU	CFU	Counts/min
	ml	*mg*									
Infective tissue culture fluid	550.0	3.55	1.9×10^7	32	16	1.60×10^4	5.3×10^6	100	100	100	100.0
Precipitate dissolved in EDTA solution	11.2	4.30	110×10^7	768	224	19.6×10^4	260×10^6	120	49	28	25.0
Pooled fractions after filtration through Sephadex column	30.0	1.90	480×10^7	326	85	6.36×10^4	250×10^6	144	56	29	21.7
Resuspended pellet after high-speed centrifugation	0.57	1.20	$1,700 \times 10^7$	8,192	6,144	80.1×10^4	$14,000 \times 10^6$	110	31	47	9.1
Band after centrifugation in sucrose density gradient[c]	4.75	0.12	80×10^7	768	640	9.98×10^4	$6,600 \times 10^6$	38	21	35	5.3

[a] The procedure outlined in Fig. 3 was used. The virus was grown in BHK cells in the presence of bovine serum albumin.

[b] Radioactivity of ^{3}H-uridine incorporated into acid-insoluble substances.

[c] Fractions from the peak of the band were pooled.

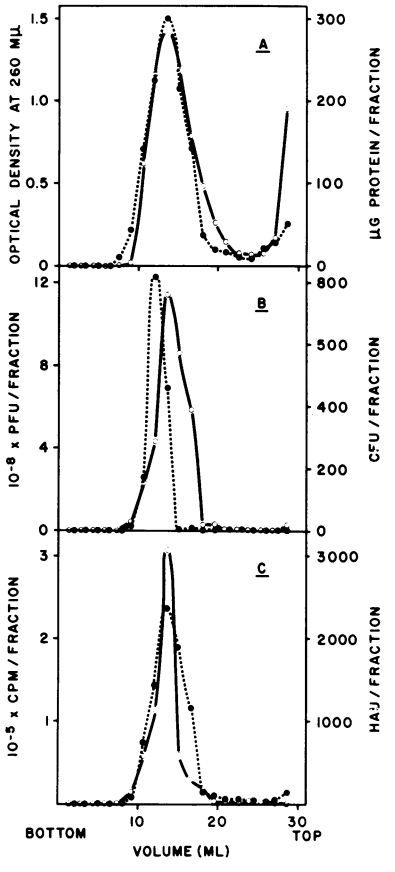

FIG. 8. *Heterogeneity of a concentrated and partially purified rabies virus (strain HEP) preparation. The virus particles were fractioned by centrifugation in a linear sucrose density gradient (10 to 55%, w/w). (A) Symbols: ○, protein content; ●, optical density at 260 nm. (B) Symbols: ○, CFU; ●, PFU. (C) Symbols: ○, HAU; ●, radioactivity of ³H-uridine. The three parts of the figure correspond to the same centrifugation experiment. The virus was grown in BHK cells in the presence of BSA.*

tion of the infectivity. A careful stepwise dialysis against solutions of decreasing sucrose concentration was necessary to retain the morphology of most of the particles.

The characteristic feature of the purified virions was the pronounced hollow core more or less evident in all particles, whether they had or had not been exposed to sucrose solution of high concentration (Fig. 5 and 10). This hollow core was rarely seen previously when cell-associated virus was studied (13). It is possible that the hollow core represents an alteration of the viral coat resulting from manipulation and does not affect the infectivity of the particles. The size of the rabies particle has been established as 180 × 75 nm (13). This sizing was done with cell-associated virus and on particles considered "complete." The present highly purified and concentrated preparations of extracellular virus showed variations in length, but not in diameter, this being uniformly 75 to 80 nm. In samples which did not exhibit any detectable heterogeneity in biological activity (when analyzed by sucrose density gradient centrifugation), the particle length was divided into three distinct groups with very few intermediates. The majority (60%) of the particles were 180 nm long. Two other groups of 150 and 125 nm were also seen, constituting 30 and 10% of the total, respectively. Variations within each group were not more than 5 nm. In preparations exhibiting heterogeneity in biological activity after fractionation in a sucrose density gradient, a similar distribution was observed, but a small proportion (up to 10%) of the particles was as short as 75 nm.

Properties of intracellular rabies virus. The procedure shown in Fig. 3 was not suitable for purification of cell-associated virus. About two-thirds of the material contained in final preparations was of host cell origin. Nevertheless, it was of interest to determine how much virus remains cell-associated 72 hr after infection, and whether the prop-

TABLE 2. *Heterogeneity with respect to various activities of rabies virus particles (strain HEP), revealed by centrifugation in sucrose density gradient*

Fraction[a]	PFU per mg of protein	HAU per mg of protein	CFU per mg of protein	PFU per counts per min[b]	HAU per counts per min	CFU per counts per min	Counts per min per mg of protein
7	180×10^7	4,000	1,000	3,700	8.2×10^{-3}	2.0×10^{-3}	5.0×10^5
8	530×10^7	5,000	1,200	8,600	8.0×10^{-3}	2.0×10^{-3}	6.1×10^5
9	250×10^7	10,100	2,800	2,000	13.0×10^{-3}	3.2×10^{-3}	8.4×10^5
10	3.2×10^7	2,400	2,400	40	3.0×10^{-3}	3.0×10^{-3}	7.9×10^5
11	3.9×10^7	1,800	2,400	46	2.2×10^{-3}	3.0×10^{-3}	8.5×10^5

[a] Fractions of the sucrose density gradient presented in Fig 8; fractions 7 and 11 correspond to the bottom and top portions of the virus band, respectively. The virus was grown in BHK cells infected at an input multiplicity of 2 PFU/cell and harvested 72 hr after infection.

[b] The virus was labeled with ³H-uridine.

TABLE 3. *Serological tests for the presence of nonviral components in purified rabies virus (strain HEP) preparations*

Antigen[a]	Reciprocal of the highest initial dilution of antigen giving positive reaction with serum against		
	Rabies virus[b]	BHK cell[b]	BSA[c]
Rabies virus....	960	4	<1
BHK cell.....	<1	128	
BSA.....	<1		1,024

[a] Undiluted preparations of purified virus, BHK cell antigen, and BSA contained 0.48, 1.96, and 9.85 mg of protein per ml, respectively.
[b] Determined in CF test.
[c] Determined by precipitation in agar gel.

erties of intracellular virus differ from those of extracellular virions. ^{3}H-uridine-labeled virus was released from 5.6×10^8 infected cells and concentrated as described in Materials and Methods. About three times less infectivity was recovered from infected cells than from a corresponding amount of infectious tissue culture fluid. It is likely that an appreciable proportion of the intracellular virus remained attached to cell debris and was removed from the suspension during clarification. The crude virus preparation was fractionated by sucrose density gradient centrifugation (Fig. 11 and 7C). Intracellular and extracellular viruses sedimented at the same rate and usually showed similar infectivity to HA and HA to CF ratios. Some preparations of intracellular virus showed, however, markedly decreased HA activity (infectivity to HA ratio about 10^7). Preparations of cell-associated virus, like those of extracellular virus, did not contain light, noninfectious HA. They did contain, however, a large amount of light CF antigen. The sedimentation pattern of components obtained by a similar procedure from the same amount of uninfected cells is also shown in Fig. 11. It indicates that the RNA-containing material banding at the same position as the rabies-specific soluble antigen is of cellular origin.

DISCUSSION

The procedures proposed thus far for the purification of rabies virus grown either in tissue of an infected animal (3, 19, 23, 27–29) or in cell culture (1, 20) have produced insufficiently purified virus in usually poor yield. The results of the present study offer an explanation for this failure. Rabies virus forms strong complexes with host cell debris, presumably cell membrane fragments, and with some serum components. This complex

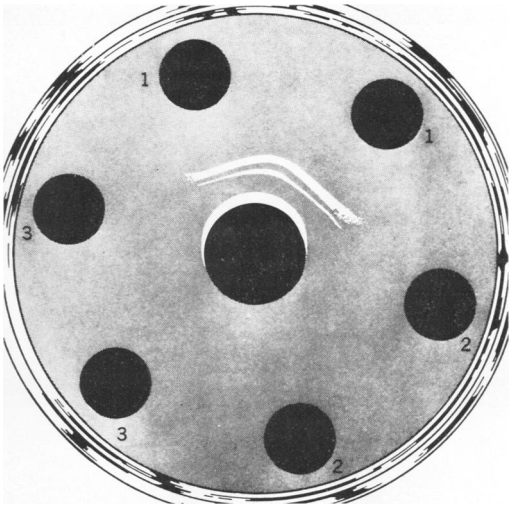

FIG. 9. *Purity of a rabies virus (strain HEP) preparation demonstrated by precipitation reaction in agar gel. Wells: center, suspension of purified virus; 1, antirabies immune serum; 2, anti-BHK cell immune serum; 3, anti-BSA immune serum.*

formation is indicated by the complete masking of the hemagglutinating activity of the infectious tissue culture fluid in the presence of serum (8*a*) and its marked inhibition in homogenates of infected cells. The formation of complexes with cell fragments can be suppressed by EDTA. When the bulk of the cell debris is removed in its presence, the hemagglutinating activity of the intracellular virus is unmasked. The binding of cell debris and of serum components leads to aggregation of the virions in concentrated preparations. Removal of aggregates during purification results in poor recovery of virus. The possibility of formation of strong complexes between rabies virus and cell or serum components should be taken into account when crude virus preparations are analyzed for heterogeneity with respect to the density or particle weight of the virions (20).

We have solved this problem by growing the virus in tissue cultures supplemented with medium containing BSA, instead of serum, and by using infectious tissue culture fluid exclusively as a source of virus. Loss of infectivity was not observed during the purification of such virus suspensions except during the last stage of the procedure, i.e., during the removal by dialysis of sucrose from the virus band collected from the sucrose density gradient. Rabies virus seems to be extremely sensitive to changes in the osmotic pressure of the environment. Rapid changes in osmotic pressure probably lead to the disruption of the outer membrane (13) of the virions and to

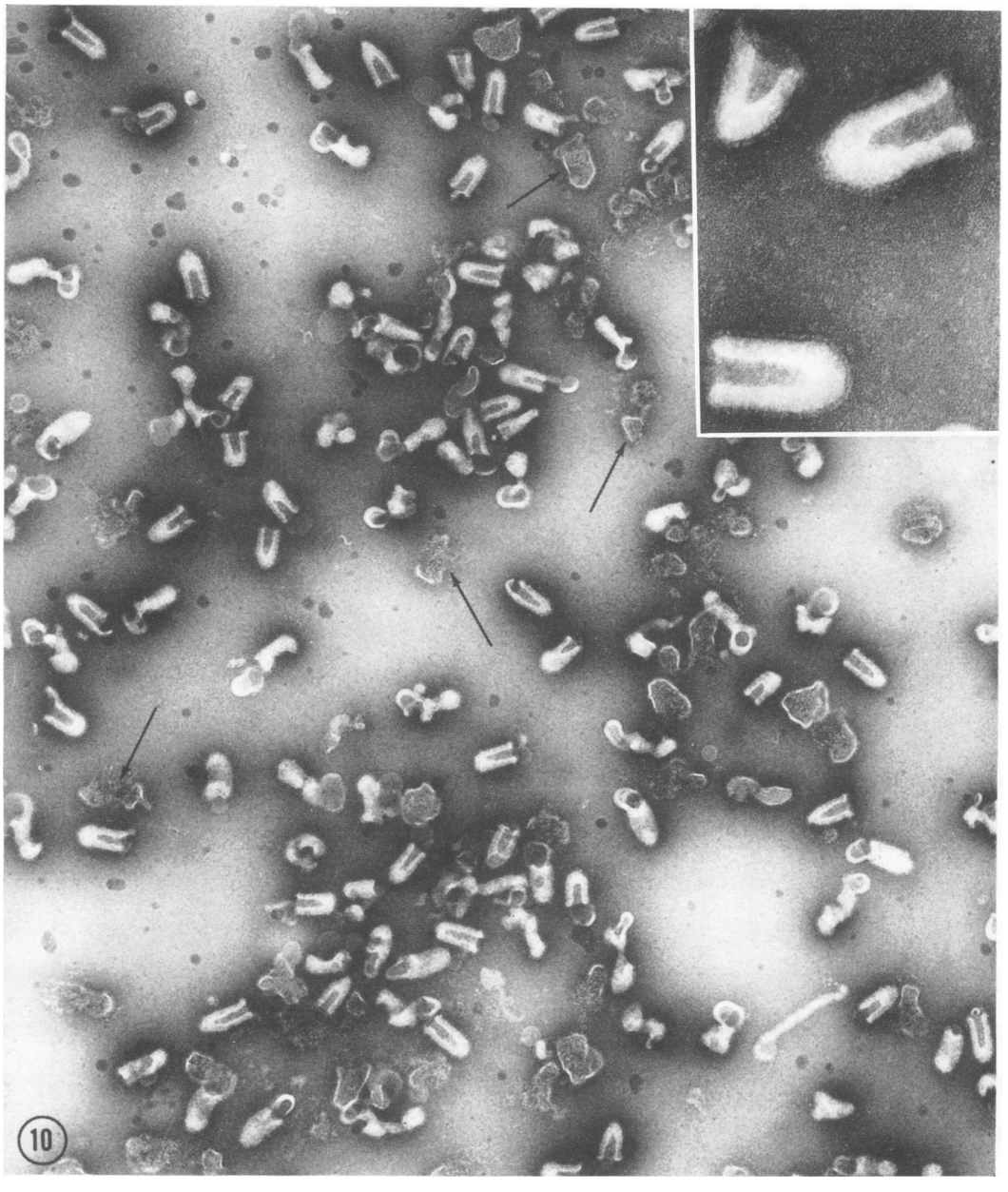

FIG. 10. *Electron micrograph of purified, extracellular rabies virus (strain HEP). The virus was grown in BHK cells in the presence of BSA and was purified by the usual procedure. After banding in density gradient, the virus suspension was freed of sucrose by dialysis. The virus was then pelleted and resuspended in NT buffer. About 20% of PFU contained in infective tissue culture fluid was recovered in such preparations. Note the partial or total breakdown of many particles (arrows). ×37,800; insert, ×123,800.*

their disintegration. Inactivation during this step was, however, markedly suppressed when the virus was dialyzed stepwise against decreasing concentrations of sucrose and when BSA or serum was added to the purified virus prior to dialysis.

Two of the methods employed for the determination of contaminants indicated that less than 5% of the material contained in the purified virus preparation was derived from the host cell. The third method, based on the extent of cross-reac-

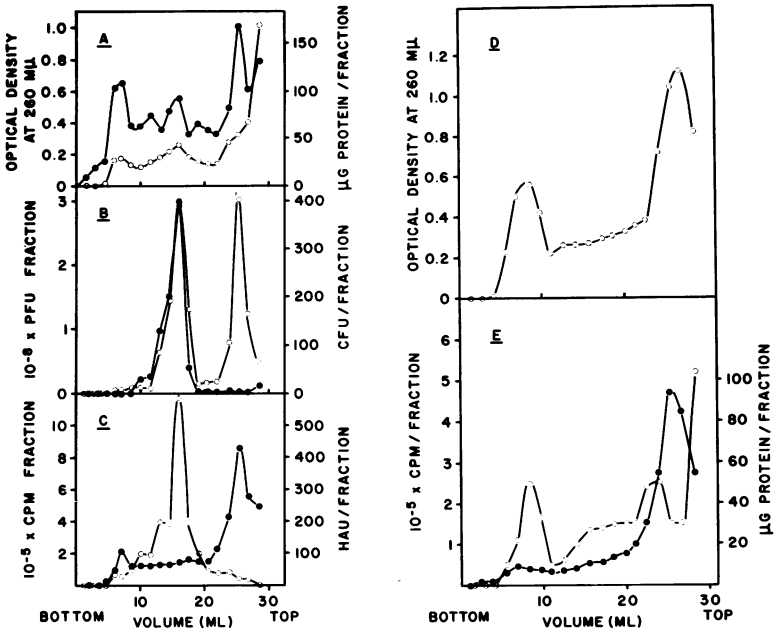

FIG. 11. *Centrifugation of a crude preparation of intracellular rabies virus (strain HEP) in a sucrose density gradient. A linear gradient of sucrose (10 to 55%, w/w) was used. (A) Symbols: ○, protein content; ●, optical density at 260 nm: (B) Symbols: ○ CFU; ●, PFU. (C) Symbols: ○, HAU; ●, radioactivity of ^{3}H-uridine. Parts A, B, and C of the figure correspond to the same centrifugation experiment. BHK cells were harvested 72 hr after infection. Parts D and E represent control experiments carried out with corresponding amounts of uninfected cells. (E) Symbols: ○, protein content; ●, radioactivity of ^{14}C-uridine.*

tion in CF tests, gave a higher estimate. In this case, the evaluation was based, however, on the unlikely assumptions that cellular components are not incorporated into the viral envelope and that all cellular antigens are equally potent in binding complement to the antigen-antibody complex. Rabies virus is released from the infected cells by budding from the cell membrane (13). Myxoviruses, which are released by the same process, have been reported to contain cellular components incorporated into their envelope (5, 22).

The majority of the virions present in purified preparations were "complete," i.e., 180 nm long. A variable proportion of the particles was shorter. The length distribution of the virus particles was similar before and after banding in sucrose density gradient, and was not altered even after rebanding of the purified virus. This fact indicated that the short particles are not artifacts formed by fragmentation of the "complete" virions during purification. Moreover, whenever virus decomposition was observed, the virions were ruptured and disintegrated into their constituents rather than fragmented into shorter bullet-shaped particles. The reason for the nonrandomness of the length distribution of the virus particles is obscure. Studies on the mechanism of maturation of rabies virus might be helpful in elucidating this phenomenon. The protein to nucleic acid ratio of the virus particles was shown to be independent of their length. This independence indicates that the amount of nucleic acid contained in the virions decreases in parallel with their length. The defectiveness of the short virus particles, sedimenting at a markedly lower rate than the "complete" virus, was shown by loss of infectivity and decreased hemagglutinating activity. For full elucidation of the relationship between the length and the biological activity of rabies virions, even more efficient methods of fractionation are needed.

High multiplicity of infection and undiluted passage in tissue culture of vesicular stomatitis virus, similar in morphology and structure to rabies virus, result in the formation of noninfectious virus particles able to interfere with the replication of infectious virus (2). The properties of these defective particles have been thoroughly studied (8, 9, 10, 11, 12). Whether the formation of noninfective rabies virus would be enhanced under similar conditions and whether the incomplete particles would be identical with the short particles described in the present study remain to be determined.

Rabies-specific HA is bound exclusively to the virions. Light, noninfective HA, similar to that found in preparations of other hemagglutinating RNA-containing viruses (7, 15, 21, 26), was detected neither in rabies virus-infected tissue culture fluid (E. Kuwert, *unpublished data*) nor in infected cells. On the other hand, both infectious tissue culture fluid (E. Kuwert, *unpublished data*) and infected cells contain light complement-fixing antigens reacting with specific sera against purified rabies virus. Experiments are in progress to characterize these "soluble" antigens.

Studies on the effect of various metabolic inhibitors on the replication of rabies virus indicate that it contains RNA (18). The results of the present study confirm this finding. Recently, single-stranded RNA was extracted from purified preparations of "complete" rabies virus and was shown to have a sedimentation coefficient of 45*S* (F. Sokol, *unpublished data*), as does the RNA extracted from complete forms of vesicular stomatitis virus (12).

Sufficient amounts of highly purified rabies virus are now available for studying the antigenic structure and the chemical composition of the virions. Electrophoresis of decomposed rabies virus in polyacrylamide gel (F. Sokol, *unpublished data*) has revealed that the protein moiety of the virus is composed of several polypeptide components.

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