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Purification of the Moloney and Rauscher Murine Leukemia Viruses by Use of Zonal Ultracentrifuge Systems

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The B-IV and B-IX zonal ultracentrifuge rotors were applied to the concentration and purification of the Moloney and Rauscher murine leukemia viruses from large volumes of infected tissue culture fluids and animal materials. Potassium tartrate, potassium citrate and sucrose gradients were used to obtain viral concentrates from the density 1.16 to 1.18 zone. Proteolytic enzyme digestion of tissue culture preparations prior to zonal ultracentrifuge processing was effective in releasing virus from cell debris and producing highly purified, though nonleukemogenic, viral concentrates. Infected Rauscher mouse plasma was processed to give highly purified infectious virus fractions. A single centrifugation of crude Rauscher mouse spleen homogenates resulted in partially purified infectious concentrates with high virus particle counts.

Zonal ultracentrifuge systems (1, 3–5) developed at the Oak Ridge National Laboratory by N. Anderson and his associates permit the application of the principle of density gradient centrifugation to the purification of viruses from relatively large volumes of crude mixtures or dilute suspensions (2, 14).

This laboratory has been concerned with the production and purification of the Moloney (11) and Rauscher (13) murine leukemia viruses from animal sources and tissue culture fluids. This report details the use of the zonal ultracentrifuge in this program, particularly the application of the instrument to the recovery of the viruses from large quantities of infected tissue-culture fluids.

MATERIALS AND METHODS

Gradient solutions. Gradient materials used with the murine leukemia viruses were chosen on the basis of infectivity experiments carried out on highly leukemogenic Rauscher mouse plasma virus as described previously (16). The factors of availability and low cost also contributed to the choices of sucrose, potassium tartrate, and potassium citrate as the principal gradient materials for these studies. Gradient materials were dissolved in 0.05 M sodium citrate, adjusted to pH 7.0 to 7.5, and cooled to 4 C before use. Gradient solutions consisted of 10 and 40% (w/w) sucrose, and solutions of potassium citrate and potassium tartrate with densities of 1.03 and 1.32. A linear relationship exists between density and refractive index for each of the gradient solutions, and experimental densities were calculated from refractive indices measured at 25 C on a Bausch & Lomb Abbe refractometer.

Operation of the B-IV ultracentrifuge batch rotor. The arrangement of the various lines for carrying out a B-IV rotor experiment is shown schematically in Fig. 1. The lines were lengths of flexible polyvinyl chloride tubing that were decontaminated and discarded after each run. The tubing connectors were Luer-Lok metal adapters and stopcocks (Becton, Dickinson & Co., Rutherford, N.J.), which were pressure-tight and yet allowed for rapid assembly and interchangeable connections. The assembled rotor, seal, and plastic tubing lines can be sterilized by ethylene oxide gassing. Sterile gradient, cushion, and overlay solutions were prepared in blood plasma bottles and connected to the appropriate tubing by use of sterile blood transfusion sets (Cutter Laboratories, Berkeley, Calif.). The gradient was pumped to the rotor edge by use of a Beckman model 131 Gradient Pump with the rotor spinning at 4,000 rev/min. For B-IV rotor studies, 1-liter linear gradients were used, ranging from 10 to 40% (w/w) sucrose, or in density from 1.03 to 1.32 for both potassium tartrate and potassium citrate.

The gradients were backed up by cushion solutions of 55% (w/w) sucrose for sucrose gradients, or of potassium citrate at density 1.35 to 1.38 for potassium tartrate and potassium citrate gradients. Cushion solutions, as well as the sample and overlay solutions, were pumped to the rotor with a Sigma-motor Model AL-4 peristaltic action pump (Sigma-motor, Inc., Middleport, N.Y.). After the rotor was filled with 1 liter of gradient and 725 ml of cushion, the sample virus suspension, ranging in volume from 20 to 500 ml, was pumped to the rotor center, displacing an equal

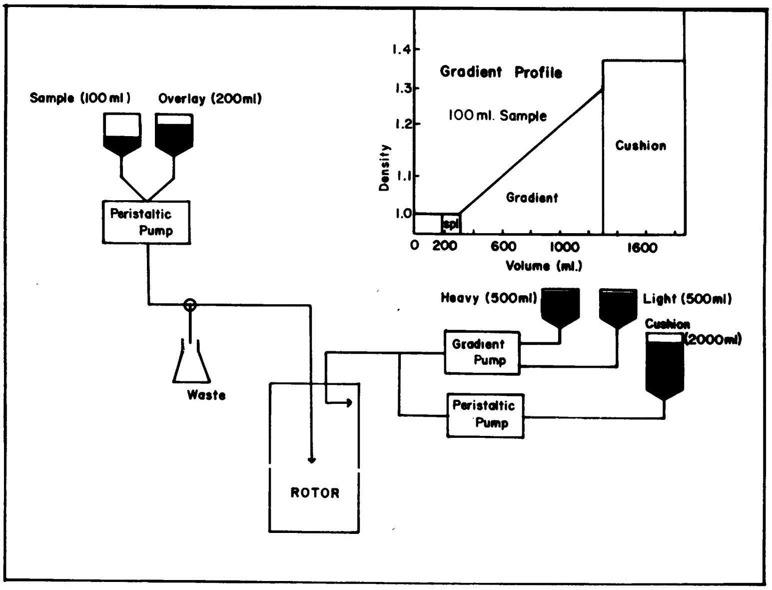

FIG. 1. *Schematic diagram of equipment and gradient profile for a zonal centrifuge run with B-IV batch rotor (dotassium tartrate or potassium citrate gradient).*

volume of cushion from the rotor edge. The sample was followed by a standard overlay solution of 200 ml of 0.03 M sodium citrate (*p*H 7) to clear the sample lines and push the sample away from the rotor core. A typical gradient profile for a B-IV run is shown in Fig. 1.

The rotor was then accelerated to operating speed, generally 28,000 to 32,000 rev/min, and maintained at operating speed for the desired time, generally 60 to 120 min. At the end of the high-speed centrifugation period, the rotor was decelerated to 4,000 rev/min, and the gradient was displaced from the rotor by pumping additional cushion solution to the rotor edge. The gradient was allowed to flow through an ultraviolet spectrophotometric monitoring system equipped with a recorder (Gilford Instrument Co., Oberlin, Ohio), and fractions of 50 or 100 ml were collected in sterile bottles.

Preparation of a fraction for electron microscopy involved dialysis of the fraction for at least 4 hr against 0.05 M sodium citrate (*p*H 7) to reduce the gradient salt concentration, followed by sedimentation of the virus in the Spinco 30 rotor at 28,000 rev/min for 60 min. Viral pellets were suspended and homogenized in 0.05 M sodium citrate (*p*H 7) to give 2- to 10-fold concentrates of the fractions. The semiquantitative virus counts were expressed as the average number of tailed virus particles per 200-mesh grid square when a standard dilution of 1 volume of sample to 2 volumes of 2% potassium phosphotungstate (*p*H 4.5) was used. It has been estimated that a concentration of 2×10^7 to 5×10^7 virus particles per ml is necessary to give an average of one virus particle per grid square by this technique (J. Monroe, *personal communication*).

Protein determinations on fractions and viral concentrates were carried out according to the method of Lowry (8) or by a modified ninhydrin procedure (7).

Operation of the B-IX continuous flow rotor. The B-IX continuous flow rotor (3; N. G. Anderson, C. L. Burger, and H. P. Barringer, Federation Proc. **23**:140, 1964) was utilized for the direct concentration of virus from large volumes of infected tissue-culture fluids (1 to 13 liters). The use of this rotor for the recovery of Moloney virus from tissue-culture supernatant fluids has been described previously (16). Briefly, in most B-IX rotor experiments, the cell-free fluids were pumped continuously at 2 to 3 liters per hr across the light side of a preloaded 500-ml gradient as the rotor rotated at 28,000 to 32,000 rev/min. At the end of the sample flow, the rotor was maintained at operating speed for an additional 20 to 30 min to sharpen the zones. The gradient then was displaced at low speed by cushion solution, and the fractions were processed in a manner similar to that described above for the B-IV rotor fractions.

In addition to the 500-ml gradients prepared with the gradient pump, equally successful B-IX runs were made with the use of "diffusion"-type gradients formed during the flow of sample through the rotor containing an initial charge of 400 ml of water and 350 ml of sucrose (40%, w/w), potassium tartrate (density, 1.32), or potassium citrate (density, 1.32). During the extended centrifugation times of 4 to 6 hr required for the processing of 9 to 13 liters of fluid, diffusion of the sucrose or salts into the water was sufficient to give satisfactory gradients of approximate linearity.

Preparation of tissue-culture fluids. In tissue culture, the Moloney virus was produced by the MT-77 infected mouse spleen cell line (10) which was grown in suspension cultures of volumes up to 10 liters (16).

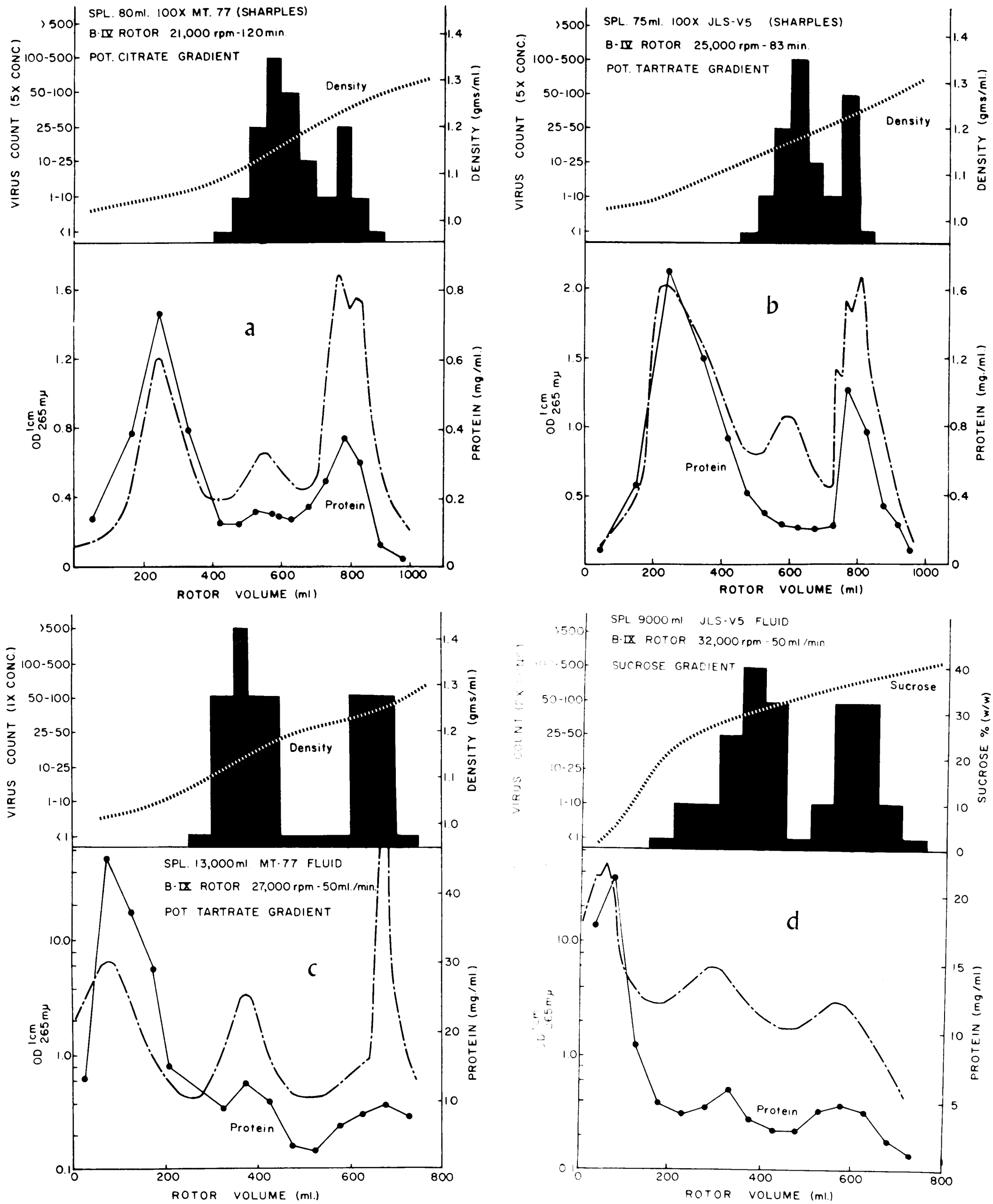

FIG. 2. *Zonal centrifuge experiments on Moloney and Rauscher tissue culture virus. (a and b) B-IV rotor runs on 100× concentrates. (c and d) B-IX rotor runs on cell-free fluid.*

The Rauscher virus was produced by the JLS-V5 mouse spleen and thymus cell line (17) which was grown in stationary culture. Virus-containing fluids were given a clarifying centrifugation at 2,000 rev/min for 20 min in an International PR-2 centrifuge to remove cells and large particulate debris. The clarified tissue culture supernatant fluids were used directly for B-IX continuous-flow rotor studies.

B-IV rotor studies with the Moloney and Rauscher tissue-culture viruses were carried out on 100-fold concentrates of the tissue-culture fluids prepared on a Servall RC-2 centrifuge or a Sharples T-1P centrifuge as previously described (16). Generally, 50 to 100 ml of a 100-fold concentrate assaying over 100 virus particles per grid square was processed in each B-IV run.

TABLE 1. *Infectivity of Moloney and Rauscher virus concentrates from zonal centrifuge runs with potassium citrate and potassium tartrate gradients*

Sample	Density	Vol	Virus Count particles/square	Bioassays, P/T[a] or average spleen wt (g)[b] at indicated dilution			
				10^0	10^{-1}	10^{-2}	10^{-3}
		ml					
Starting MT-77, 100×	—	80	100–500	6/7	5/5	5/5	2/6
Fraction 11, 3×	1.164	20	100–500	7/8	8/8	4/7	3/7
Fraction 16, 3×	1.230	20	25–50	5/7	5/8	4/5	0/7
Starting JLS-V5, 100×	—	60	100–500	6/6	4/5	5/5	1/6
JLS-V5, 100× treated with 0.25% Protease P-6, 36 C, 20 min[c]	—	60	100–500	0/8	0/9	0/10	0/8
Fraction 12, 4×	1.165	12	>500	0/8	0/9	0/9	0/9
Starting Rauscher plasma, 0.5×	—	200	50–100[d]		2.504	1.087	
Fraction 13, 3×	1.150	17	<1		0.174	0.122	
Fraction 14, 3×	1.165	17	>500		2.020	1.760	
Fraction 15, 3×	1.182	17	>500		1.350	0.936	
Starting Rauscher spleen homogenate 50% (w/w)	—	150	—		1.987		
Fraction 9, 7×	1.132	7	1–10	0.152			
Fraction 10, 7×	1.148	7	25–50	0.191			
Fraction 11, 7×	1.165	7	100–500	1.152			
Fraction 12, 7×	1.185	7	100–500	1.676			
Fraction 13, 7×	1.204	7	1–10	0.117			
Fraction 14, 7×	1.224	7	1–10	0.135			

[a] P/T, positive/total at 120 days.
[b] Average spleen weight of 8 to 10 mice at 21 days.
[c] Zonal centrifuge sample.
[d] Estimated.

Proteolytic enzyme digestion of the 100-fold concentrates prior to density gradient centrifugation was employed for further purification of the tissue-culture viruses. The 100-fold concentrates were incubated at 34 to 36 C for 20 to 30 min in the presence of 0.25 to 0.50% ficin (Nutritional Biochemicals Corp., Cleveland, Ohio), or Protease P-6 (Chas. Pfizer & Co., Inc., New York, N.Y.), before layering the sample on the gradient in the B-IV rotor.

Preparation of animal materials. Weanling BALB/c mice were inoculated with a 10^{-2} dilution of highly infectious Rauscher mouse plasma, and the animals were sacrificed and bled 28 days after infection. The blood was collected into an equal volume of 0.30 M potassium citrate and clarified by low-speed centrifugation to remove the cells. B-IV rotor studies were made on 200 to 400 ml of 0.5× plasma per run.

The density gradient technique was also applied to the spleens obtained from the Rauscher-infected BALB/c mice sacrificed for the production of plasma. Twenty-five to fifty spleens, averaging about 2 g each, were homogenized for 60 sec in 0.05 M sodium citrate (*p*H 7), by use of a Lourdes Model MM homogenizer, to give 25 to 50% (w/w) suspensions. The homogenates were clarified in the PR-2 centrifuge at 2,000 rev/min for 20 min, and the supernatant fluids were used as zonal centrifuge samples.

Bioassays. Moloney virus preparations were bioassayed in 48- to 72-hr-old BALB/c mice and observed up to 4 months for evidence of leukemia. Rauscher virus samples were inoculated into 25- to 28-day old weanling BALB/c mice and were observed up to 4 months postinoculation for evidence of leukemia. Generally, at least 10 mice were inoculated intraperitoneally with 0.1 ml of sample. Development of palpable splenomegaly confirmed at autopsy was used as the index of infection. In experiments with highly infectious Rauscher virus from animal sources, the spleen weight assay technique (6) was used.

TABLE 2. *Recovery of Moloney virus from particulate debris zone by homogenization and recentrifugation on second gradient*

Sample	Volume	Density	Virus count (particles/grid square)
	ml		
Starting MT-77, 100×	40	—	100–500
First centrifugation			
Fraction 9, 3×	20	1.168	50–100
Fraction 15, 3×	20	1.232	25–50
Second centrifugation of homogenized fraction 15			
Fraction 10, 7×	7	1.170	25–50
Fraction 15, 7×	7	1.243	10–25

TABLE 3. *Protein content of Moloney and Rauscher virus concentrates from zonal centrifuge experiments*

Rotor	Sample	Virus count (particles/grid square)	Protein
			µg/ml
B-IV	*Tissue Culture Virus*		
	Starting MT-77 or JLS-V5 100X concentrates	100–500	5,000–8,000
	Virus concentrate from density 1.16 to 1.18 zone (non-enzyme-treated)	100–500	500–1,000
	Virus concentrate from density 1.16 to 1.18 zone (enzyme-treated)	100–500	100–200
	Mouse Plasma Virus		
	Starting Rauscher plasma, 0.5X	50–100[a]	6,000
	Virus concentrate from density 1.16 to 1.18 zone	>500	100–150
	Mouse Spleen Virus		
	Starting Rauscher spleen homogenate, 50% (w/w)	—[b]	>50,000
	Virus concentrate from density 1.16 to 1.18 zone	100–500	4,000
B-IX	*Tissue Culture Virus*		
	Starting MT-77 or JLS-V5 cell-free infected fluid	1–5	30,000–40,000
	Virus concentrate from density 1.16 to 1.18 zone	100–500	800–1,200

[a] Estimated count. Virus count on 0.5X plasma impossible because of high salt concentration. Sedimented Rauscher plasma, 10X, assayed > 500 particles/square.

[b] Virus count impossible because of high debris level.

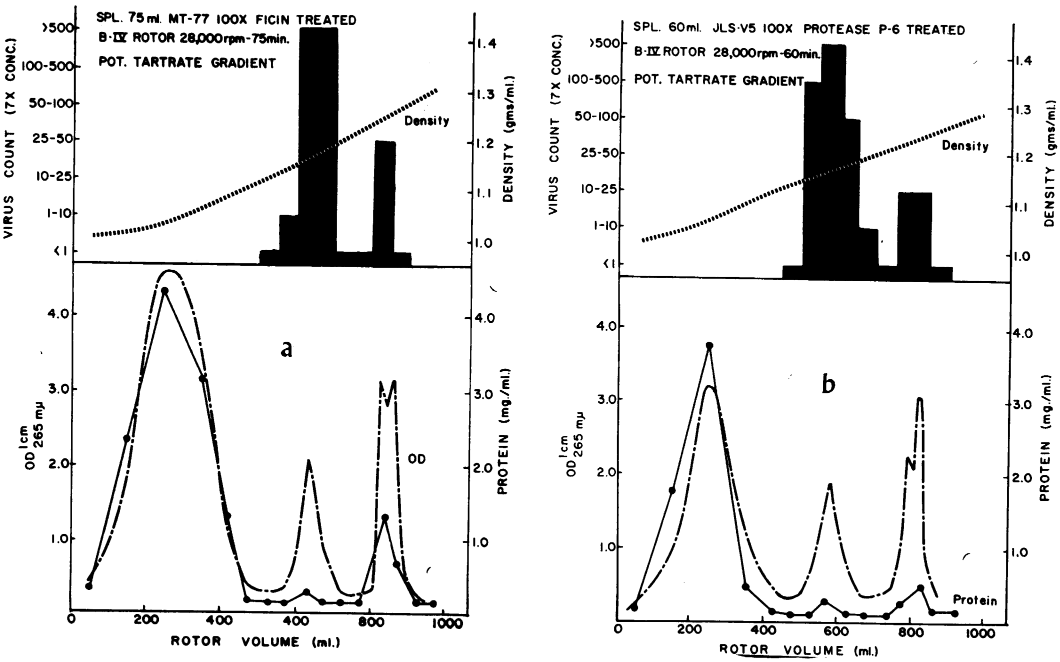

FIG. 3. *Zonal centrifuge experiments on proteolytic enzyme-treated Moloney and Rauscher tissue culture virus. (a) MT-77. (b) JLS-V5.*

RESULTS

Tissue-culture virus. The Moloney and Rauscher viruses have been shown to possess a similar buoyant density of 1.16 in potassium citrate (12). This density is intermediate between the two major groups of contaminating materials present in the infected tissue culture fluids: the soluble protein, lipid, and other low-density or slow-sedimenting components; and the membranous and other particulate impurities that band in the density range of 1.23 to 1.25. The results of typical B-IV and B-IX rotor experiments with Moloney and Rauscher tissue culture materials are shown in Fig. 2. They clearly indicate the separation of large amounts of the murine leukemia viruses into the zone of density 1.16 to 1.18 for potassium citrate and potassium tartrate gradients, and 25 to 30% (w/w) sucrose for sucrose gradients under the centrifugation conditions used in these experiments. Significant quantities of virus were found also in the particulate debris zone at density 1.23 to 1.25 for potassium citrate and potassium tartrate gradients, and 35 to 40% (w/w) sucrose for sucrose gradients. Bioassays indicated that the virus from both zones was leukemogenic (Table 1 and reference 16), and electron microscopy indicated no significant differences in virus morphology at the two density levels. When samples of the viral concentrates from the particulate debris zone were thoroughly homogenized (or digested with proteolytic enzyme, or both; see below) and recentrifuged on a second gradient, considerable quantities of virus were recovered at density 1.16 to 1.18 (Table 2). This indicated that the virus which banded initially in the particulate debris zone was not a unique population, but was virus entrapped by or attached to membranous debris.

Virus concentrates from the zone at density 1.16 to 1.18 from B-IV and B-IX runs on tissue culture preparations were considerably more purified compared with starting materials, as judged by the ratio of virus count to protein content (Table 3). However, analysis of these virus concentrates by isopycnic gradient centrifugation, with the use of potassium tartrate gradients and centrifugation times of 3 hr in a Spinco SW 25.1 rotor (9), revealed that the virus concentrates generally were contaminated with traces of lighter and heavier particulate matter.

The treatment of MT-77 or JLS-V5-fold concentrates with the proteolytic enzymes ficin or Protease P-6 prior to density gradient centrifugation resulted in sharp, highly purified virus bands at density 1.16 to 1.18 (Fig. 3). The virus concentrates from the density 1.16 to 1.18 zone from enzyme-treated samples generally had higher virus counts with lower protein values than the comparable concentrates from untreated preparations (Table 3). Also, as shown in Fig. 3, the virus counts were low in the particulate debris fractions from runs on enzyme-digested preparations, indicating a substantial release of entrapped virus from the membranous debris. Isopycnic gradient centrifugation of the virus concentrates from enzyme-treated preparations almost always resulted in a single sharp virus band at a density of 1.16. Electron micrographs also confirmed that these concentrates were relatively free from contaminating particulate impurities (Fig. 4). However, bioassays of these purified virus preparations were essentially negative with respect to leukemogenic activity in BALB/c mice (Table 1), although the preparations have been shown to be antigenically active (15).

Animal specimens. Plasma from BALB/c mice infected with Rauscher virus was processed in the B-IV rotor; typical results are shown in Fig. 6.

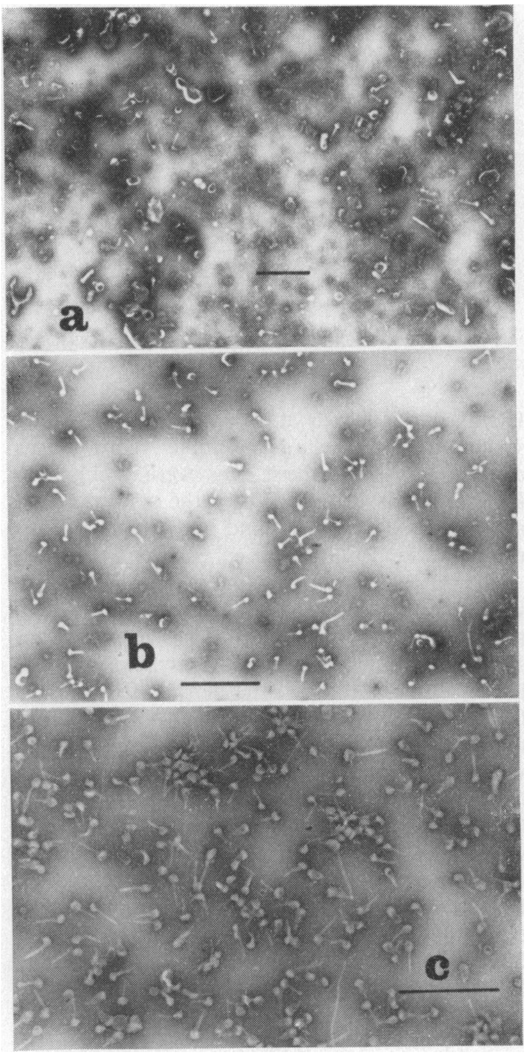

FIG. 4. *Electron micrographs of Rauscher virus concentrates after processing on the zonal centrifuge. Source of virus: (a) Rauscher BALB/c mouse spleen; (b) Rauscher JLS-V5 enzyme-treated; (c) Rauscher BALB/c mouse plasma. The bar in each micrograph represents 1 μ.*

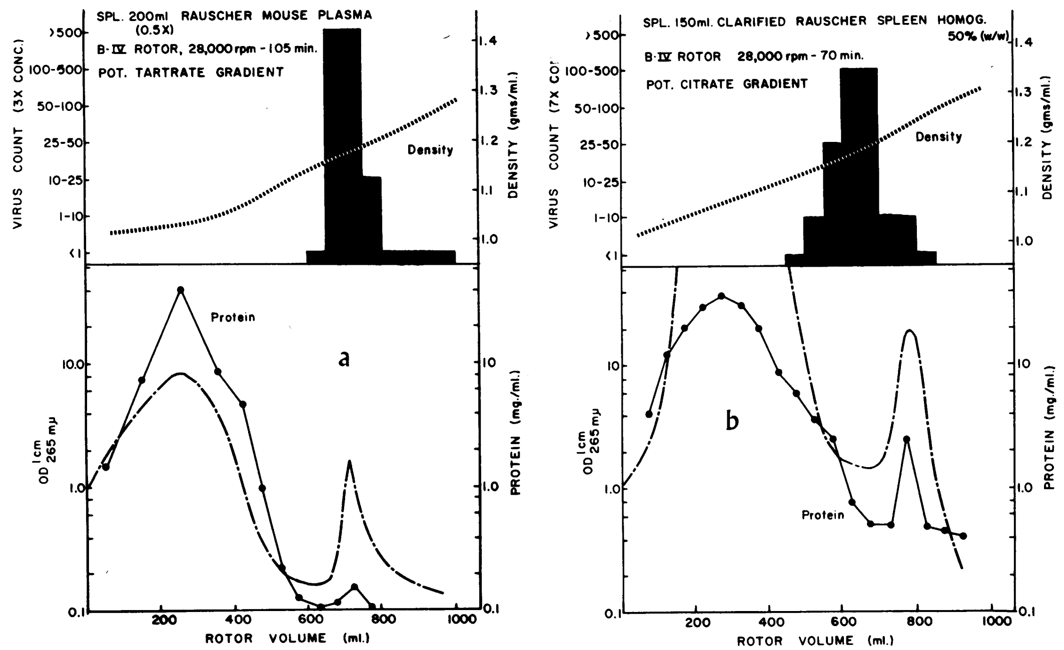

FIG. 5. *Zonal centrifuge experiments on Rauscher virus from animal sources. (a) Infected BALB/c mouse plasma. (b) infected BALB/c mouse spleen.*

Highly purified virus at a density of 1.16 readily separated from a large "soluble" zone. Virus concentrates from these runs were highly leukemogenic, as determined by the spleen weight assay method (Table 1), and had a high virus to protein ratio (Table 3).

Zonal centrifuge experiments on Rauscher spleen homogenates were made to test the effectiveness of the density gradient technique in separating murine leukemia virus from a crude tissue source. Although the density 1.16 to 1.18 fractions from these experiments were contaminated with much cellular debris, high levels of virus were easily observed by electron microscopy (Fig. 4 and 5). Virus counts on the starting clarified spleen homogenates by electron microscopy were impossible because of the crude nature of the samples. Again, protein assays indicated the extent of virus purification (Table 3), and bioassays by the spleen weight assay method showed a good correlation of infectivity with virus count (Table 1).

DISCUSSION

The results obtained in these studies with the zonal centrifuge indicate the usefulness of this instrument for the concentration and fractionation of the Moloney and Rauscher murine leukemia viruses from large volumes of crude tissue culture fluids and animal specimens. With the B-IX rotor, the viruses were concentrated about 100-fold and purified about 30-fold from cell-free unconcentrated tissue culture fluids (Table 3). Rauscher-infected mouse plasma was concentrated over 5-fold and purified about 50-fold by use of the B-IV rotor. The effectiveness of the density gradient centrifugation technique in separating virus from a highly impure source was shown by the fractionation of Rauscher-infected mouse spleen homogenates into bands with high virus concentrations.

Limited infectivity studies indicate that sucrose, potassium tartrate, and potassium citrate gradients can be used for zonal centrifuge studies with these viruses, with the preservation of substantial leukemogenic activity in the virus concentrates. Proteolytic enzyme digestion of tissue culture virus preparations prior to zonal centrifuge processing resulted in highly purified, antigenically active virus fractions, but these fractions showed a substantial loss of infectivity.

It should be noted that these studies were aided by the fact that the murine leukemia viruses are relatively low in buoyant density. The viruses conveniently fall in a density zone between the "soluble" components and the bulk of particulate cellular debris. The application of the zonal centrifuge to the isolation of intracellular viruses with densities overlapping those of the major cellular contaminants would require extensive study by the so-called S-ρ system proposed by Anderson (3): sedimentation rate separations in the zonal centrifuge followed by isopycnic banding in angle-head rotor tubes.

From the mechanical point of view, the zonal centrifuge has proved to be a reliable instrument to operate. This report is a summary of over 200 experimental runs ranging from 15 min to 6 hr of high-speed centrifugation time, with as many as three runs in a 16-hr day, including complete breakdown and reassembly of the rotors and accessory lines.

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