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Purification of Large Quantities of Influenza Virus by Density Gradient Centrifugation

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New zonal centrifuges can conveniently process as much as five orders of magnitude (10^5) greater sample volumes than conventional swinging-bucket rotors. The continuous-sample-flow-with-banding versions may be used in series with ancillary purification procedures. Here we have studied the combined process: absorption and elution of influenza virus with barium sulfate followed by concentration and isopycnic banding of the virus in a buffered sucrose gradient. Kilogram quantities of impurity have been rapidly separated from grams of purified virus, which have been conveniently concentrated several hundred-fold by the purification process. Experimental vaccines made by these procedures are being evaluated.

By using rate-zonal centrifugation followed by isopycnic banding, we previously demonstrated that commercial influenza virus vaccine could be purified an additional 10-fold and that the protective antigens were separated from most of the pyrogens usually found in such partially purified material (14). To increase the amount of antigen processed with zonal centrifuges, various ancillary procedures for concentrating and purifying the virus were also reported (15). In parallel with these studies, new continuous-flow zonal centrifuge systems capable of handling much larger volumes have been developed (1-7, 18). This paper reports some of our current collaborative results on the application of these systems to large-scale influenza virus purification.

The results demonstrate the utility of the barium sulfate absorption-elution technique (9, 11, 13) when employed with zonal centrifugation. Many of the procedures devised are applicable to the problem of isolating other viruses in sufficient quantity for analytical, immunochemical, or vaccine purposes.

MATERIALS AND METHODS

Virus. Influenza virus was produced by standard techniques (Division of Biologics Standards, National Institutes of Health, USPHS, Minimum Requirements: Influenza Virus Vaccines, Types A and B; and Public Health Service Regulations, Title 42, Part 73) in the allantoic cavity of 11-day-old embryonated chicken eggs. The strains used in this study were: A/PR-8, A_1/Ann Arbor/1-57, A_2Taiwan/1-64, A_2/Japan-170/62, B/Maryland/1-59, and B/Massachusetts/3-66.

Barium sulfate absorption-elution. Prior to zonal centrifugation, virus was concentrated 3.5 times and partially purified by the barium sulfate, absorption-elution procedure modified as follows:

Virus was absorbed from allantoic fluid (*p*H 7.8 to 8.0) at 0 to 3 C by the addition of potassium oxalate and dry barium sulfate as shown in Table 1. The suspension was stirred for 90 min and allowed to stand overnight. Harvesting of the barium sulfate with absorbed virus was done in a 4-liter bowl Sharples continuous-flow centrifuge at 7,000 rev/min with a flow rate of 50 to 80 liters/hr.

Virus was eluted by resuspending the barium sulfate into one-seventh the original volume of fluid having a composition shown in Table 1. On the following day, the barium sulfate was removed from the resuspended virus at room temperature by centrifugation at 800 $\times$ *g* for 10 min in an International PR-2 centrifuge. The final eluate containing the partially purified virus was adjusted to *p*H 8.0 and diluted approximately twofold with pyrogen-free distilled water to a final specific gravity of 1.050 $\pm$ 0.005.

Zonal centrifugation. Two distinct classes of rotors were used. The first was used for rate-zonal centrifugation of concentrated virus and included the B-IV (available from the Spinco Division of Beckman Instruments, Palo Alto, Calif.; reference 4) and B-XV (fabricated at the Oak Ridge Gaseous Diffusion

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TABLE 1. *Amounts of dry barium sulfate used to adsorb six strains of influenza virus and the composition of the eluting media to remove the virus from the barium sulfate*

Strain	Adsorption		Elution					
	$BaSO_2$ added per liter of allantoic fluid	Oxalate	Bone gelatin	Na_3 citrate	*p*H	NaCl	Tris	Tween 80
	g	*M*	%	*M*		*M*	*M*	*M*
PR-8	40	None	0.2	0.25	7.2	None	None	None
Taiwan	50	0.16	0.2	0.25	7.2	1.0	0.4	0.16
Ann Arbor	50	0.16	0.2	0.25	7.2	1.0	0.4	0.16
Jap 170	50	0.16	0.2	0.25	8.8	1.0	0.4	0.002
B Mass	50	0.16	0.2	0.25	7.2	1.0	0.4	0.16
B Md	60	0.16	0.2	0.25	7.2	1.0	0.4	None

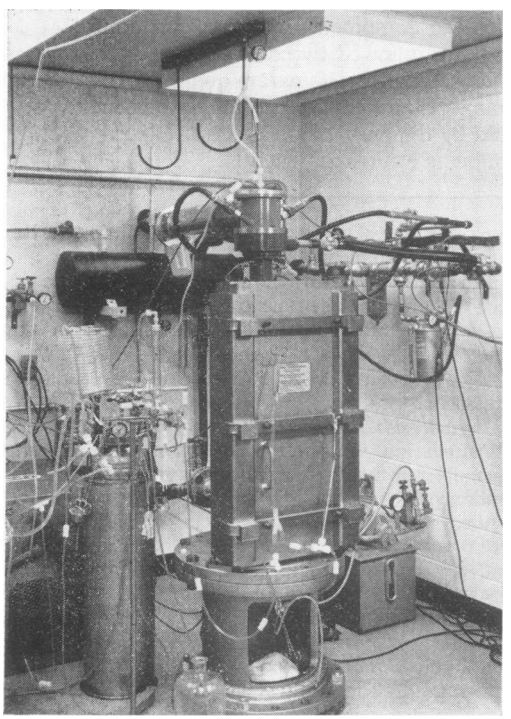

FIG. 1. *K-II zonal centrifuge.*

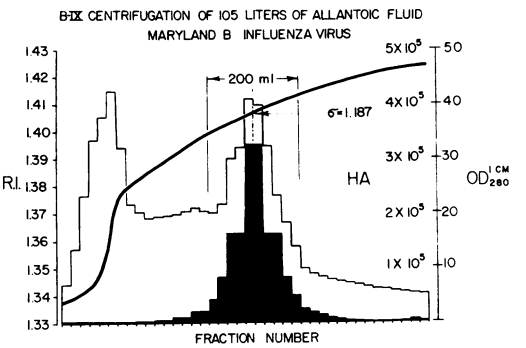

FIG. 2. *Distribution of influenza virus (HA shaded area) and optical density in a sucrose gradient (refractive index) from a B-IX rotor. See text for details.*

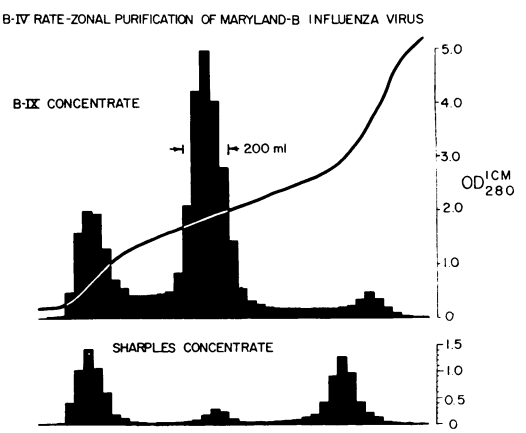

FIG. 3. *Rate zonal sedimentation profiles for the B-IX concentrate of Fig. 2, and a 10× Sharples commercial virus concentrate. The central peak contains the virus.*

Plant; reference 6) rotors used in a modified Spinco zonal ultracentrifuge.

The second class of rotors concentrated and purified virus by the continuous-flow-with-banding process (1, 5). The B-IX rotor used here was an experimental prototype and was modified repeatedly during the course of this study, culminating in the present B-XVI rotor (8). The K-II centrifuge (Fig. 1) used for these studies was a prototype air-driven centrifuge designed and constructed at Oak Ridge as a continuous-flow-with-banding zonal centrifuge suitable for large-capacity production (Anderson, Waters, Nunley, Cline, and Gibson, *in preparation*). Although the principles and manner of operation of this centrifuge are identical to those of previous Oak Ridge continuous-flow zonal centrifuges, certain important differences are noted here.

TABLE 2. *Pyrogenicity of a standard commercial monovalent strain of PR-8 influenza virus: fractionated in B-XV rotor*

Sample	Vol	CCA/ml	Total CCA	Protein	Total protein	Pyrogenicity[a]		
						1:4	1:40	1:400
	ml			*mg/ml*	*mg*	*C*	*C*	*C*
Original	350	1,940	680,000 (100%)	0.644	225 (100%)	+1.02	+0.90	+0.38
Soluble impurities	440	148	65,000 (9.6%)	0.164	72 (32%)	+0.98	+0.62	+0.17
Purified virus pool	480	716	343,000 (50.4%)	0.040	19 (8.5%)	+0.92	+0.39	+0.05
Particulate impurities	720	<68	<49,000 (7.2%)	0.088	63 (28%)	+0.42	+0.40	−0.29

[a] Mean temperature rise of three rabbits during first 3 hr at the three dilutions given.

TABLE 3. *Comparison of purity and concentration of several experimental vaccines*

Sample	Volume concentration factor	Concn (CCA units/ml)	CCA concentration factor	Lowery protein (mg/ml)	Purity (CCA units/ml of protein)	Purification factor
Original allantoic fluid	1×	145	1×	10.5	13.8	1×
Sharples purified	10×	1,164	8×	0.64	1,820	132×
B-IX purified	525×	43,350	300×	6.1	4,250	318×
B-IV purified	210×	9,000	62×	1.1	8,500	618×

TABLE 4. *Mouse protection test, purified monovalent (Md.B) influenza vaccine*

Vaccine	Original vaccine concn (CCA/ml)	LD_{50} challenge dose	No. of mice surviving/no. of mice inoculated with vaccine dilution				
			1:5	1:25	1:125	1:625	1:3,125
B-IX purified	933	40	6/6	6/6	6/6	5/6	1/6
B-IV purified	898	40	6/6	6/6	6/6	3/6	0/6
NIH reference	200 (as Md.B)	40	6/6	6/6	2/6	1/6	0/6

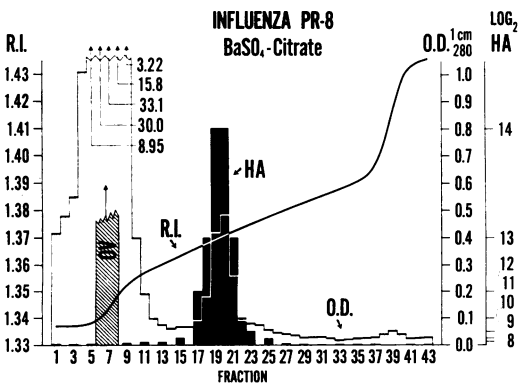

FIG. 4. *Rate zonal sedimentation profile for influenza virus concentrated by the $BaSO_4$ absorption-elution technique. Initial zone (OV) was 100 ml, optical density 1 cm/280 = 48.0; log_2 HA = 16.*

The reorienting gradient technique (2) for initially loading the gradient and for terminally unloading the fractions while the K-II rotor was at rest was used exclusively. Initially the K-II rotor (3.6-liter internal volume) and associated tubing was filled with the lighter of the two solutions which were used to make the diffusion gradient. For our gradients, we used sucrose solutions, buffered with 0.01 M phosphate and 0.02% gelatin at *p*H 8.0. The sucrose concentration in the lighter of the two gradient solutions was adjusted so that this solution's specific gravity was 0.005 g/cc higher than the specific gravity of the virus eluate (ca. 1.050) from the $BaSO_4$.

After the rotor was completely filled with light solution, it was accelerated from rest to 2,000 rev/min, and the direction of fluid flow through the rotor was reversed several times to remove entrapped air. The rotor was then brought to rest by means of the air brake. An appropriate amount (usually about 1.8 liters) of the heavier of the two gradient solutions [60% (w/w) sucrose, buffered as described for the

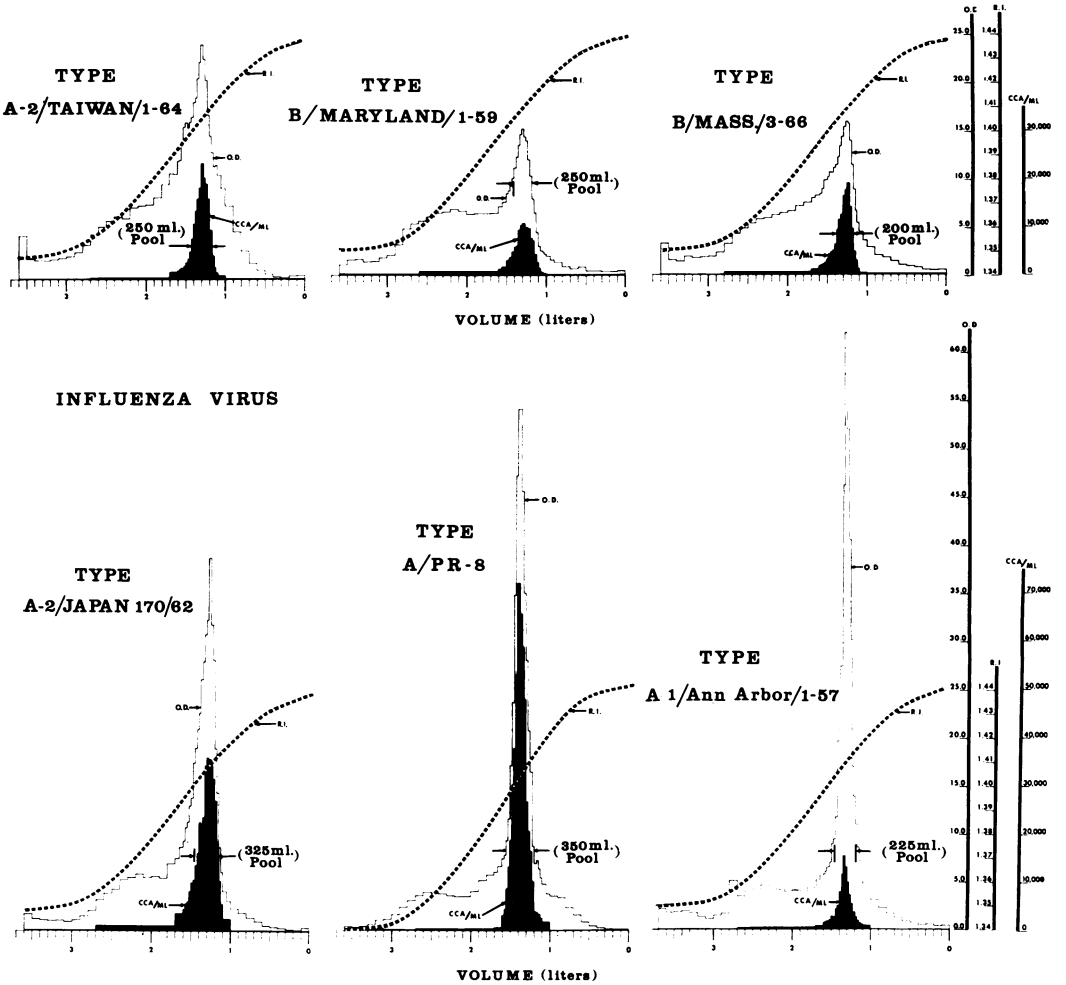

FIG. 5. *Hemmagglutinin and optical density profiles of influenza virus purified by the combine process: $BaSO_4$ absorption-elution, followed by isopycnic banding in the K-II zonal ultracentrifuge.*

light solution] was then forced into the bottom of the rotor, displacing an equal volume of the lighter solution from the top. (Note that the K-II rotor is unique in having liquid seals and flow lines attached to both ends of the rotor.) The lower rotor inlet line was then closed and the rotor was smoothly accelerated to 2,000 rev/min to reorient the liquid density gradient from a vertical to a centrifugal direction. Fluid flow inboard of the imprisoned gradient was then established, from the top to the bottom of the rotor, and maintained while the rotor was smoothly accelerated to 20,000 rev/min (400 rev/min^2 to 4,000 rev/min; maximal acceleration rate, to 20,000 rev/min). Virus sample flow was then started while the rotor was further accelerated at a rate manually governed to prevent high hydrostatic back pressure (not exceeding 15 psi) in the input line. This back pressure is due to the presence of two fluids which differ in density by approximately 0.005 g/cc in the 700-ml core taper volume. Displacement of a dense fluid by a light one results in a back pressure in a continuous-flow rotor where both fluid lines are brought back to the axis of rotation before leaving the rotor. Once 700 ml had been displaced from the rotor by the lighter virus sample, the rotor was accelerated to operational speed without developing hydrostatic pressure in the input line.

Generally, the K-II centrifuge was operated at 27,000 rev/min with 75 ft^3 of filtered dry air per min at 25 psi. (The maximal operating speed of the K-II centrifuge with an aluminum rotor will probably be about 30% higher, depending on the development and evaluation of safety shielding studies now in progress. Presently, the virus-laden stream flows a distance of 76 cm in the rotor where the average force field is approximately 37,000 $\times$ g. At the higher

TABLE 5. *Influenza virus purification by combined $BaSO_4$ absorption-elution, followed by isopycnic banding in the K-II centrifuge*

Strain	Lot no.	Input			Output			
		Vol of allantoic fluid (liters)	Virus concn (CCA/ml	Total input (CCA × 10^6)	Total output (CCA × 10^6)	Overall yield (%)	No. of 100 CCA doses per harvested egg	Purity (CCA/mg of protein)
Md.B	T-61160	89.5	159	14.2	3.70	26.0	4.0	22,300
	T-61164	93.0	128	11.9	4.12	34.6	4.2	18,020
	T-61166	152.0	62	9.44	3.18	33.7	2.1	8,820
Taiwan	T-61162	145.0	152	22.0	4.40	20.0	3.2	8,380
	T-61165	78.0	148	11.55	1.74	15.0	2.5	20,100
	T-61167	81.0	129	10.50	2.36	22.4	2.9	8,580
Ann Arbor	T-61163	150.8	200	30.2	2.60	8.6	1.9	15,770
	T-61168	103.7	132	13.7	2.56	18.7	2.7	8,580
	T-61171	95.0	193	18.3	2.12	11.6	2.4	8,480
PR-8	T-61161	56.7	323	18.3	7.29	39.8	11.9	10,500
	T-61169	144.0	200	25.6	15.90	62.0	13.6	13,400
	T-61170	99.3	314	31.2	13.30	42.7	13.7	8,680

operating speeds contemplated, the centrifugal force would be approximately 70% higher.) Acceptable clean-out of influenza virus occurred from the 1.050 specific gravity citrate solutions we employed when the sample flow was adjusted to between 4 and 5 liters/hr.

Upon termination of flow, sample fluid remaining in the core taper volume (700 ml) was displaced by a sucrose gradient solution 0.005 g/cc lighter than the sample. Rotation was then continued at full speed to allow virus to band for an additional 30 min.

The rotor was allowed to coast to rest before it was unloaded. The mass of the rotor is sufficiently large to insure an even and gradual reorientation during deceleration. The gradient was collected as a discrete series of fractions from the bottom of the rotor by use of air pressure through the upper fluid line.

Gradient shape. Gradient shape was determined by measuring the sucrose concentration with a Bausch and Lomb Abbé refractometer.

Absorbancy. Absorbancy between 250 and 350 nm was determined for all fractions in a Cary 15 recording spectrophotometer.

Chicken cell agglutination (CCA) assays and mouse protection tests. These were performed as previously described.

Protein determinations. Protein was determined by the method of Lowry (12).

Hemagglutinin assays. Hemagglutinin assays were done with microtiter equipment (17) or alternatively in plastic trays as follows: a 0.5-ml sample was serially diluted in twofold increments with buffered saline, and a washed chicken red cell suspension (0.7%) was added to each cup. After a 1-hr incubation period at room temperature, the trays were inclined at approximately 45°. The end point was read as the dilution where the red cells remain in a button in the bottom of the cup for at least 15 sec without migrating down the wall.

Immunodiffusion. Immunodiffusion was performed with a NIL-Saravis apparatus (obtained from the National Instrument Laboratories, Inc.) with Celotate membranes (Millipore Corp., Bedford, Mass.). At the end of a 72- to 90-hr incubation period followed by fixing and rinsing, the membrane was stained using Coomassie Brilliant Blue (10), except that the drying of the membranes was done at 37 C for 45 min. Rabbit antisera against chicken serum was obtained from the Colorado Serum Co., Denver, Colo. The membrane was cleared with light paraffin oil, mounted between glass plates, sealed with Permount, bound together with tape, and photographed.

RESULTS

Studies with the B-IX rotor. A 105-liter volume of allantoic fluid containing Maryland B strain of influenza virus was processed through the B-IX rotor. Figure 2 shows the optical density and hemagglutinin profiles of fractions harvested from this continuous-flow isopycnic banding device. This crude egg harvest had been first cleared of gross debris in the Sharples Supercentrifuge with essentially all of the virus remaining in the flowing stream. This virus was then quantitatively captured in the B-IX rotor spinning at 40,000 rev/min with a flow rate of 8 liters/hr. The bulk of soluble impurities remained in the stream flowing through the B-IX rotor and was discarded. As calculated from either the hemag-

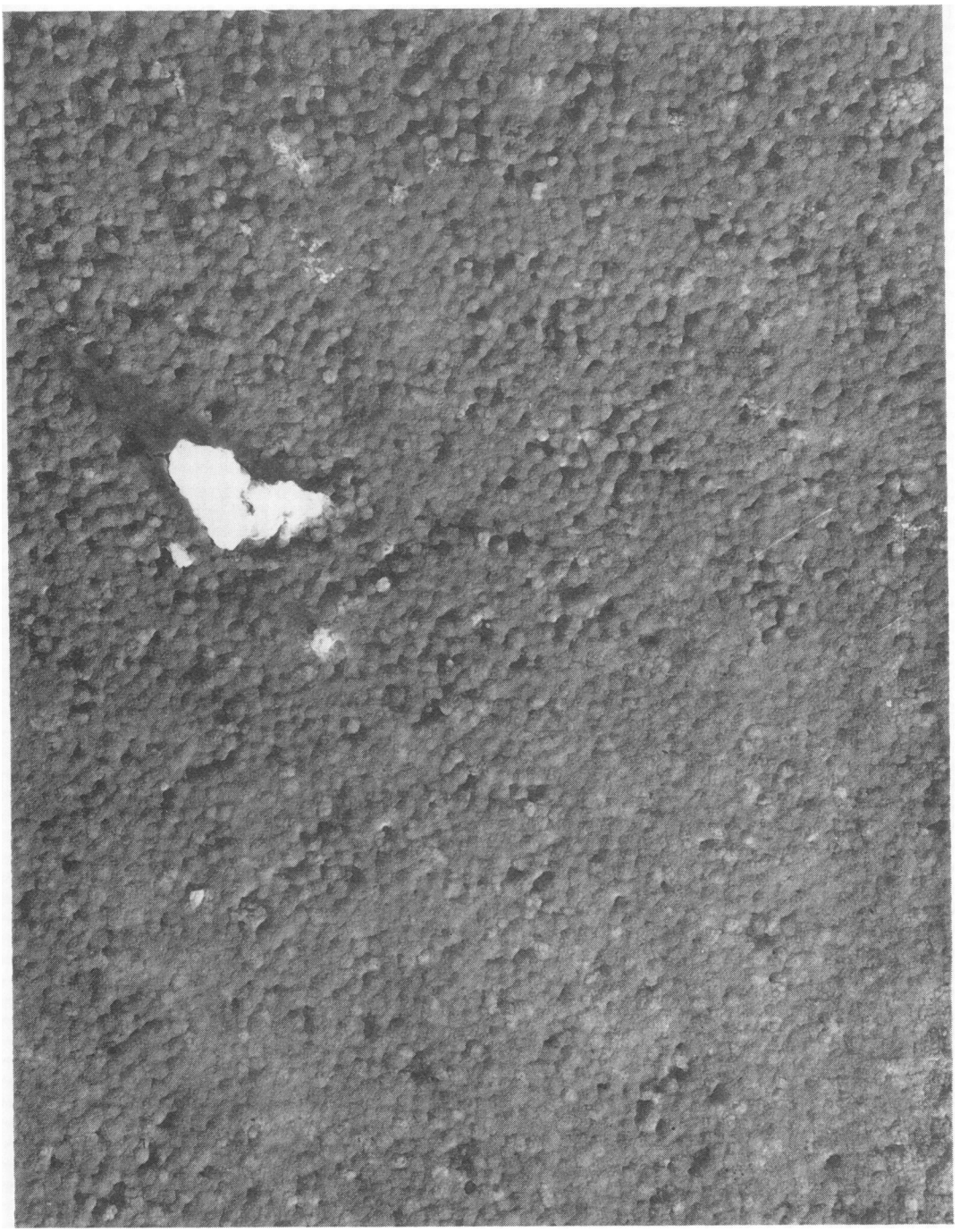

FIG. 6. *Electron micrograph of a monovalent vaccine (Md.B) purified by the combined process.* ×*37,200.*

glutinin test or the chick cell agglutination (CCA) assay, two-thirds of the total virus originally present in the 105-liter pool of crude allantoic fluid was recovered in the sucrose gradient as a band having a volume of 200 ml. The density of the peak fraction was 1.187 g/cc. The peak hemagglutinin titer was 320,000, as compared with 512 for the original allantoic fluid.

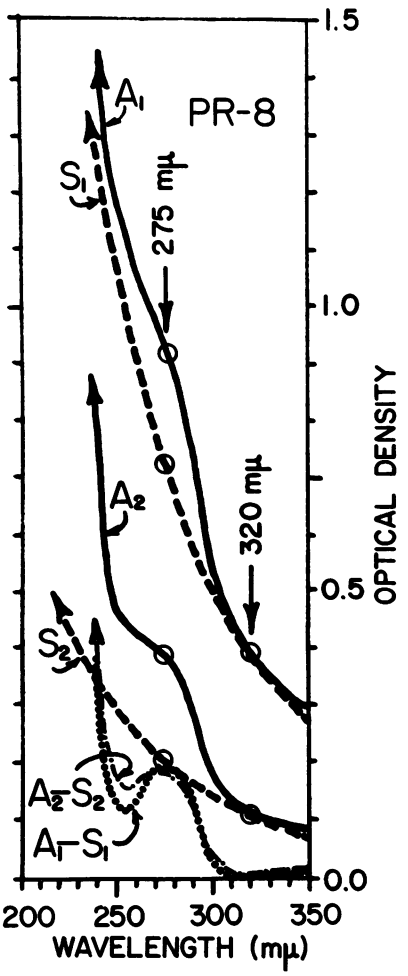

FIG. 7. *Ultraviolet-absorption spectra for purified influenza virus, A/PR-8 (1.0-cm cell against water blank). Curve A_1 : total absorbancy of a rate-zonal purified pool after dilution fivefold with distilled water; final refractive index, 1.3438. Curve S_1 : theoretical absorbancy due to an object which scatters inversely with the fourth power of the wavelength (Rayleigh scattering), assuming entire absorbancy at 320 mμ due to scatter. Curve A_2 : total absorbancy of the same pool as in A_1, except that pool was diluted fivefold with optically transparent 60% (w/w) sucrose; final refractive index, 1.4245. Curve S_2 : same as S_1 except that S_2 equals entire absorbancy of A_2 at 320 mμ.*

An 80-ml amount of this B-IX concentrate was further purified by a rate-zonal process in the B-IV rotor after first removing the sucrose in a G-25 Sephadex column. The rate-zonal sedimentation profile of the B-IX concentrate was compared with a similar profile for a typical commercial 10X vaccine concentrate as shown in Fig. 3. Soluble impurities are found in the peak on the left; large particulate impurities, in the peak on the right. The central peak, rising out of a background of small particles, contains the virus. The relation between the size of the virus peak and the impurity peaks demonstrates graphically the increased purity of the virus from

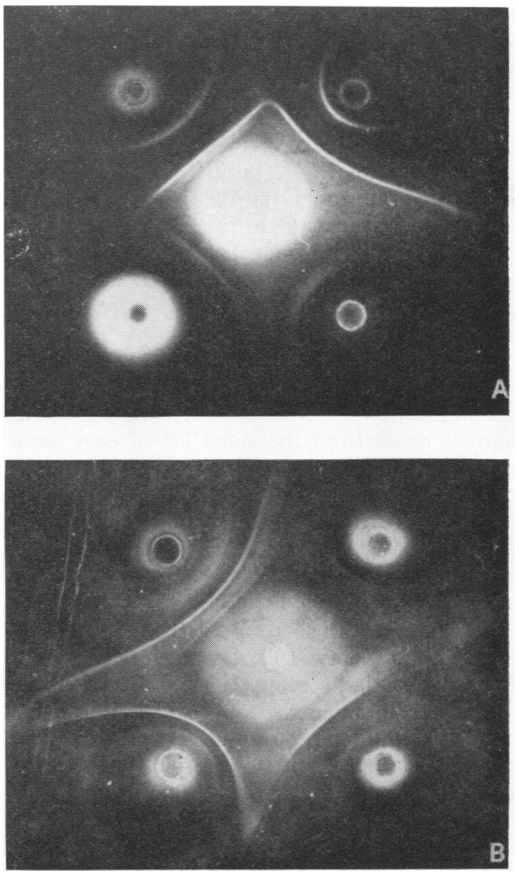

FIG. 8. *Comparison of commercial and purified influenza vaccines by immunodiffusion. A, upper left: commercial bivalent vaccine. A, upper right: commercial polyvalent vaccine. A, lower left: purified bivalent vaccine. A, lower right: purified polyvalent vaccine. B, upper right: purified trivalent vaccine. B, remaining peripheral wells: commercial polyvalent vaccines from three different manufacturers. In each case, the center well contains rabbit serum against chicken serum. The commercial vaccines contain a total of 600 CCA/ml: the purified bivalent and polyvalent vaccines, 1,200 CCA/ml. The bivalent vaccines contained A_2/Taiwan/1-64 and B/Maryland/1-59. The polyvalent vaccines had, in addition, A/PR-8 and A-1/Ann Arbor/1-57; and, in Fig. 8B, A_2/Japan 170/62. The purified trivalent vaccine had the 1967–68 "Bivalent" formula: A_2/Taiwan/1-64, A_2/Japan 170/62, and B-Massachusetts/3-66.*

the B-IX process. Dilutions of material taken from various peak regions of a similar rate-zonal fractionation of 10× commercial vaccine concentrate were tested for pyrogenicity in rabbits. Table 2 shows that much of the pyrogen is present with the soluble macromolecular impurity, and was separable from the immunizing fraction by the rate-zonal technique. Based on the dilution giving approximately equal pyrogenicity, the purified virus fraction was one-tenth as pyrogenic as the commercial product from which it came. Very little pyrogen was associated with the large particulate impurity of this lot.

Virus from the B-IX purified and virus from the B-IV rate-zonal purified derivative was diluted, inactivated with Formalin, and sterilized by filtration through HA membranes (Millipore Corp.). These vaccines passed all control testing necessary for vaccine production for human use.

Table 3 gives a tabular comparison of the original crude allantoic fluid, B-IX and B-IV vaccines, and a standard 10X Sharples concentrate made from the same lot of eggs, as a control. Table 4 shows the results of a mouse potency test of these zonal centrifuge purified vaccines, as performed under standard conditions. It is seen that both of these experimental monovalent Maryland B vaccines protected half the mice even when diluted 624-fold.

Essentially identical results were obtained for similar monovalent A_2/ Taiwan vaccines. However, difficulty has been often experienced with the B-IX rotor in attempts to repeat these results routinely with volumes of allantoic fluid as large as 100 liters. Large particulate impurities, present in variable amount in large volumes of allantoic fluid, interfered with sedimentation by overloading the gradient and by plugging the small channels in the rotor and in the rotating seal, preventing steady-state flow, or terminal unloading of the fractions. (Less difficulty was encountered with 100-liter volumes of tissue culture fluid in the B-IX rotor.)

This problem has been largely solved by first absorbing the virus from allantoic fluid with $BaSO_4$, followed by elution with citrate solutions. This process is more efficient for separating the virus from large particulate impurities than other methods we have examined (15), as shown by the rate-zonal sedimentation profile in Fig, 4. The adsorption and continuous-flow centrifugation steps are complementary, the first removing large particulate material, and the second eliminating soluble impurities.

For production purposes, the K-II centrifuge has now superseded the B-IX and B-XVI. Figure 5 shows the hemagglutinin and optical density profiles for six strains of influenza virus with the use of the combined process and the K-IIB centrifuge. These are representative profiles from commercial production-sized lots, which utilized an average of 15,000 eggs each. These virus pools were diluted and further processed through Formalin inactivation and bacteriological filtration to make monovalent and polyvalent vaccines. Table 5 lists some of the overall yield and purity data for 12 lots of monovalent vaccine. The sources of losses are shown in Table 6. Table 7 lists mouse potency data for a $BaSO_4$—K-II polyvalent vaccine. After the A and B potency tests were made, this vaccine was found to be inadvertently contaminated with a staphylococcus. It was then sterilized by HA membrane filtration. The C and D tests showed that potency was not lost by this process. Figure 6 shows an electron micrograph of an experimental vaccine made with the combined process.

Portions of the K-II virus pools were further purified by rate zonal centrifugation in the B-XV rotor as previously described. The repurified virus, generally recovered in 25% sucrose, usually contained between 1,000 and 2,000 CCA units and 50 to 100 μg of protein per optical density

TABLE 6. *Source of loss, in per cent of total CCA originally present in allontoic fluid*[a]

Strain	Overall avg yield of product	Lost unabsorbed to $BaSO_4$	Lost uneleuted from $BaSO_4$	Lost in K-II effluent	Lost in K-II gradient, outside of virus band	Lost during inactivation and filtration	Lost to assay sampling	Unaccounted for loss
Md.B	31.4	−15.7	+6.3	−29.8	−16.8	+0.8	−10.0	−3.4
Taiwan	19.1	−25.2	−28.3	−22.7	−11.1	+2.5	−10.0	+13.9
Ann Arbor	13.0	−11.2	−37.0	−28.4	−11.0	+3.4	−10.0	+7.2
PR-8	48.2	−20.2	−0.2	−17.1	−6.7	+4.2	−10.0	+2.4
Average	27.9	−18.1	−14.8	−24.5	−11.4	+2.7	−10.0	+4.0

[a] Minus equals a loss; plus equals a gain.

unit at 280 mμ in a 1-cm cell. A typical ultraviolet-absorption curve for this highly purified material, with and without λ^{-4} scatter correction is given in Fig. 7. Figure 8 shows immunodiffusion patterns obtained with rabbit antichicken serum in the center well and highly purified vaccine or commercial vaccine in the peripheral wells.

DISCUSSION

Centrifugation in density gradients, which has been customarily performed in swinging buckets with milliliter quantities of sample and centiliter amounts of gradient, has proved most useful in virology and in other areas as an analytical (and small-scale preparative) technique. The B-IV and B-XV batch type zonal rotors, with total volumes of 1.7 liters, utilizing a deciliter or greater sample volume, extends the preparative possibility of zonal techniques by two orders of magnitude. The continuous-flow, isopycnic banding B-IX rotor (total volume, 750 ml) permits optimal processing of liters or tens of liters of sample to add two or even three more orders of magnitude to the volumes conveniently processed. The larger K-II centrifuge (volume, 3.6 liters), when used sequentially with other purification procedures, has routinely processed more than 150-liter volumes of allantoic fluid per day. To date, 175 K-II runs have been made with only minor operating problems. This is approximately 1,300 hr at 27,000 rev/min using two instruments. Kilogram quantities of impurity have been rapidly separated from gram quantities of purified virus, which has been conveniently concentrated several hundredfold by the purification process.

We anticipate that some of the losses listed in Table 6 will be brought under better control. For example, the large sampling losses (10%) for these prototype runs can be diminished. Most of the virus loss in the K-II gradient (11.4%) was arbitrarily left in the shoulders by selecting out a relatively narrow band centered on the virus peak. Some of the virus lost in the K-II effluent (24.5%) could have been captured by using a higher rotor speed, a slower flow rate, or possibly by a more efficient core design. Likewise, with further study, improved yields (32.9% loss) from the $BaSO_4$ process are anticipated.

The mouse potency ratios listed in Table 7 represent the factor by which a purified vaccine was diluted in excess of the National Institutes of Health reference standard vaccine to confer equal protection (50% survival for challenged mice). A possible explanation for this enhanced potency in mice is that we underestimate the amount of antigen in these highly purified vaccines. This would happen if the purified virus aggregated irreversibly, causing an unequal distribution of the absolute number of virus particles during the serial dilutions used to measure the virus (CCA test). An aggregate of many virus particles may also be no more efficient than a single virus particle in sticking two red cells together. We have electron microscopic evidence for some virus aggregation. The consequences of such aggregation would be that: (i) some loss of antigen during processing is artifactual and does not represent loss of immunizing mass; (ii) the purity is underestimated; (iii) residual toxicity relates to a higher dose than indicated by the CCA test because we underestimate the amount of antigen given.

TABLE 7. *Mouse potency ratios for a $BaSO_4$-K-II purified influenza vaccine*

Strain	Concn (CCA/ml)	Potency ratio: exptl vaccine/NIH reference			
		Test A	Test B	Test C	Test D
PR-8	165	11.03	5.71	9.87	8.67
Ann Arbor	110	13.49	5.01	3.35	6.59
Md.B	300	8.69	8.65	5.70	25.12
Taiwan	225	2.56	2.95	1.70	3.35
Jap 170	300	1.00	1.29	1.14	3.40

Alternatively, the mouse may respond more efficiently to a highly purified virus vaccine than to an impure preparation.

Light scattering of the purified virus accounts for a large fraction of the observed absorption in the ultraviolet. Since such scattering depends on both the amount of virus present and on the refractive index of the suspending medium, care must be exercised if absorbance data are used as quantitative purity criteria in influenza virus purification in density (and refractive index) gradients. The clinical evaluation of these vaccines, which is now in process, will be reported elsewhere (F. B. Peck, Jr., et al., *in preparation*).

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