

# Continuous-Flow Ultracentrifugation of Canine Distemper Virus and Infectious Canine Hepatitis Virus

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The use of a Spinco L-4 zonal centrifuge with the B-XVI continuous-flow rotor for the purification of canine distemper and infectious canine hepatitis viruses is described. Up to 68 liters of virus was processed at one time. Infectious canine hepatitis virus was found to band at 39% sucrose and canine distemper virus banded between 32 and 48% sucrose. The virus was concentrated 10-fold, and the purity of the virus, as measured by protein concentration, was increased by 99%.

Continuous-flow zonal centrifugation has been applied to the purification of influenza virus (12), rhinoviruses (6), adenovirus (4), polyoma virus, and Semliki Forest virus (5). Although there is some evidence that the zonal centrifuged virus provides a better vaccine, the primary goal of centrifugation is a safer vaccine free from extraneous materials.

This report describes the purification of infectious canine hepatitis (ICH) virus and canine distemper virus (CDV) by continuous-flow zonal centrifugation in a sucrose gradient.

## MATERIALS AND METHODS

**Virus culture.** Secondary dog kidney cells were grown in monolayers in 5-liter Povitsky flasks at 37 C with Hanks balanced salt solution supplemented with 10% fetal calf serum and 1% lactalbumin hydrolysate. After establishment of the monolayer, the growth medium was decanted and replaced with maintenance medium containing the Cornell-1 strain of ICH. After 75 to 95% of the cell sheet showed cytopathic effect (CPE), usually at 3 to 5 days, the virus was harvested into plastic jugs and stored at -90 C.

CDV, Rockborne strain, was grown on secondary dog kidney cells and harvested in the same manner as ICH virus.

**Assay.** ICH virus was assayed in Leighton tubes by using secondary dog kidney cells. The tubes were planted with dog kidney cells and incubated at 37 C with Hanks balanced salt solution supplemented with 10% fetal calf serum plus 1% lactalbumin hydrolysate. After an 80% cell sheet was established, the growth medium was removed and the tubes were inoculated with 2 ml of serially diluted virus. A 5-ml amount of maintenance medium (medium 199, Gibco) was added

to the tubes. The cell sheets were observed after 48 and 72 hr. The titer was calculated by the Reed-Muench formula (11).

CDV was assayed by using fluorescent-antibody techniques with conjugate derived from hyperimmune dogs as described by Liu (7).

**Zonal centrifugation.** The viruses were banded isopycally in a Spinco B-XVI zonal rotor. From 10 to 68 liters of virus in tissue culture medium was purified at one time. The rotor, rotating seal, and tubing were sterilized by ethylene oxide. Aseptic conditions were maintained throughout the processing of the virus.

The rotor was filled with a sterile linear sucrose gradient by using preformed cams and a Beckman gradient pump. The gradient was monitored by using an Abbe type refractometer to insure linearity. The gradients used in these procedures extended from 5 to 50% (w/w) sucrose. Pumping was continued until all air was removed from the rotor and seal. In the second step, the line from the center of the rotor was attached to a pressurized 5-gal can (Millipore Corp., Bedford, Mass.) and the virus was forced through the rotor at 1 to 2 ml/min by using filtered nitrogen while the rotor was accelerated to 40,000 rev/min. When the rotor reached 40,000 rev/min, the virus flow rate was increased to the predetermined optimum flow rate. The flow rate was measured by a shielded flow meter (Gelman Instrument Co., Ann Arbor, Mich.) that had been calibrated for the tissue culture medium. A clamp was incorporated in the line to the flow meter, and 2 psi of back pressure was maintained against the rotating seal to minimize cross-leakage between the influent and effluent streams.

**Determination of optimum flow rate of medium.** When the rotor attained 40,000 rev/min, the flow of effluent was adjusted to 1 liter/hr. After the flow had stabilized, a sample of the effluent was collected for assay.

The flow rate was adjusted by increasing the nitrogen pressure and was again allowed to stabilize. Another sample was collected, and the procedure was repeated until enough samples had been collected to determine the amount of virus in the effluent at varying flow rates.

**Isopycnic banding.** When the rotor attained 40,000 rev/min, the nitrogen pressure was increased to give the optimum flow rate. After all the medium had passed through the rotor, the lines were flushed with phosphate buffer (0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7.2) to remove any remaining medium.

The rotor was allowed to spin for 1 hr to insure isopycnic banding of the virus and then was decelerated to 2,000 rev/min.

Sterile 60% sucrose was pumped to the outside of the rotor at a rate of 15 ml/min. The displaced rotor contents were collected in sterile 40-ml fractions and frozen at  $-90^\circ\text{C}$  until assayed.

Protein concentration was determined by the method of Lowry et al. (8). An Abbe type refractometer was used to determine the per cent of sucrose of each sample.

Virus purity was estimated by an immunodiffusion technique (3). Three healthy rabbits (3.5 kg) were injected subcutaneously with the impure virus preparation containing 2 mg of protein/ml. The rabbits were injected with 1 ml every other day for a total of nine injections. After 1 week, the rabbits were injected subcutaneously with 1 ml of the nonpurified virus mixed with 1 ml of Freund's complete adjuvant. The rabbits were allowed to rest for 1 week and were

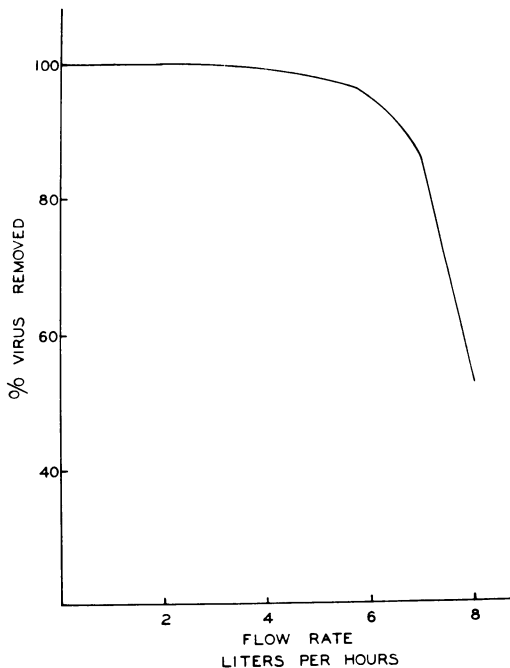


FIG. 1. Clean out of infectivity from ICH virus harvest in the B-XVI rotor at 40,000 rev/min as a function of flow rate.

TABLE 1. Clean out of infectivity of infectious canine hepatitis and canine distemper viruses<sup>a</sup>

Flow rate <sup>b</sup>	Clean out <sup>c</sup> ICH virus (%)	Clean out <sup>c</sup> CDV (%)
1	100	99
2	100	99
3	99	99
3.2	99	100
3.4	99	97
3.6	94	98
3.8	97	96
4.0	98	94
4.4	95	92
4.8	95	78
5.2	92	64
5.5	84	
6.1	88	
6.7	80	
7.4	74	

<sup>a</sup> Each value is an average of three experiments.

<sup>b</sup> Flow rate is expressed as liters per hour.

<sup>c</sup> The removal of virus from harvest calculated from the difference in  $\text{TCID}_{50}/\text{ml}$  between the harvest and the effluent from the rotor.

then bled, and the serum was collected. The above procedure was repeated using nonpurified CDV.

## RESULTS

**ICH virus.** Figure 1 illustrates the effect of flow rate on retention of ICH virus in the B-XVI rotor. At flow rates lower than 4.8 liters/hr, 95% of the infectivity was removed. Therefore, during all studies a flow rate of about 4.5 liters/hr was used. Table 1 shows the data for determining virus "clean out" for both ICH and CDV.

Table 2 shows the results of an isopycnic separation of ICH virus by using a sucrose density gradient and the B-XVI continuous flow rotor. The viral activity of the original sample was 7.2 log. The isopycnicly banded material contained virus with a titer of 8.2 log. The virus banded at about 39% sucrose concentration. This is an approximate density of 1.2 g/cm<sup>3</sup>. Because of the apparent low density of the virus, electron micrographs were prepared with purified virus negatively stained with 2% phosphotungstic acid. Essentially all of the virus particles took up the stain, indicating a defect of the coat. However, these defective particles were capable of infecting and conferring immunity. Whether the defective coat is a property of the attenuated ICH virus strain has not been determined. The optical density of the fractions was read at 260 nm on material diluted 1/100 or 1/10.

In addition to absorbance, protein was determined on the collected fractions (8). The

protein concentration of the original material was 12.0 mg/ml. The protein concentration of the fractions collected from the rotor ranged from 2 mg/ml (fraction 1) to 80  $\mu$ g/ml (fraction 16, the fraction containing the virus). These

TABLE 2. Combined continuous-flow centrifugation and isopycnic banding in the B-XVI rotor<sup>a</sup>

Fraction no. <sup>b</sup>	Log viral activity <sup>c</sup>	Optical density at 260 nm	Sucrose <sup>d</sup>
1	5.0	6.0	8
2	5.0	6.0	11
3	5.0	6.0	12
4	5.0	6.0	15
5	5.0	6.0	17
6	5.3	6.0	19
7	5.3	5.0	20
8	5.3	4.0	23
9	5.3	4.0	25
10	5.4	4.0	26
11	5.4	1.0	31
12	6.4	1.0	35
13	7.2	1.0	36
14	8.2	0.5	39
15	6.2	0.5	45
16	6.0	0.1	54
17	6.0	0.1	59
18	6.0	0.1	60
19	6.0	0.1	60
20	6.0	0.1	60

<sup>a</sup> Each point represents three runs of 20 liters each.

<sup>b</sup> Fractions contain 40 ml.

<sup>c</sup> Expressed as TCID<sub>50</sub>/ml.

<sup>d</sup> Expressed as per cent (w/w) at 4 C.

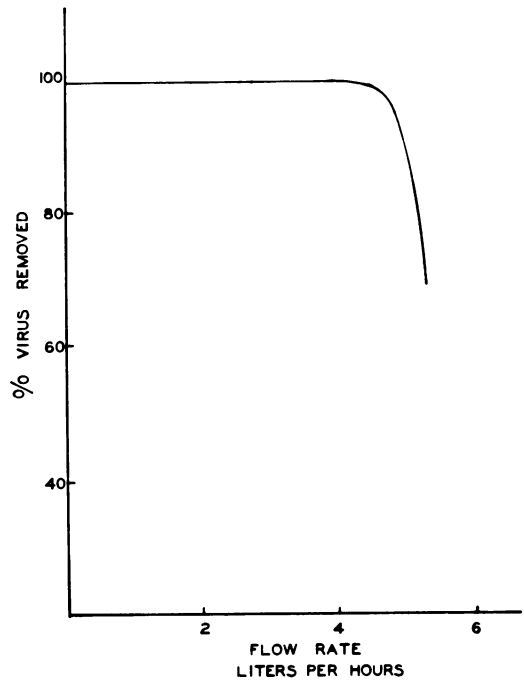


FIG. 3. Clean out of infectivity from CDV harvest in the B-XVI rotor at 40,000 rev/min as a function of flow rate.

figures are an average of three separate runs using 10 liters of harvested virus per run.

Thus the virus band contained only  $2.5 \times 10^{-3}\%$  of the protein originally present. The degree of purification between fraction 1, which

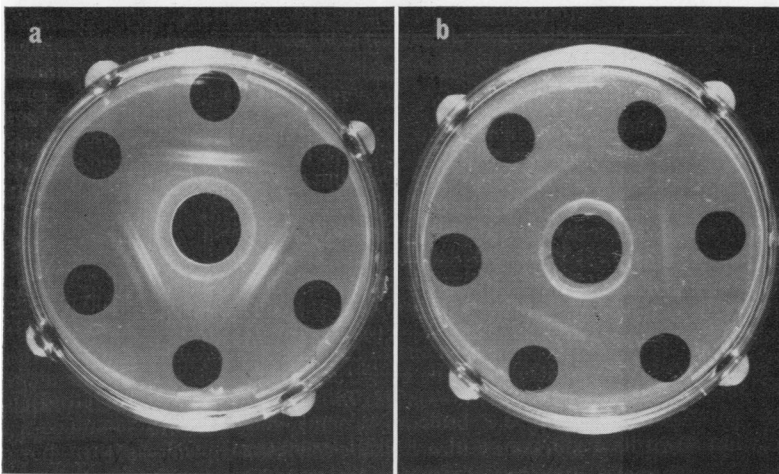


FIG. 2. Comparison of nonpurified and purified ICH virus by immunodiffusion. (a) Nonpurified virus in alternate outer wells. (b) Purified virus in alternate outer wells. In each case the center well contains rabbit serum against nonpurified virus.

contains soluble protein, and the virus band is 96%.

Figure 2a shows the results of the immunodiffusion of nonpurified ICH virus and nonpurified ICH virus antisera. Figure 2b shows the precipitin bands obtained with purified virus and antiserum to the nonpurified virus.

**CDV.** Figure 3 demonstrates the effect of flow rate on the retention of CDV in the B-XVI rotor. The optimum flow rate is about 3.8 liters/hr (Table 1).

To facilitate the assay of CDV (Table 3), only three fractions were cut from the gradient which extended from 5 to 50% sucrose (w/w). The original virus had a titer of 4.4 log. The effluent contained 2.3 log CDV/ml. The first 300 ml of the gradient and overlay contained 2.0 log of virus. The second 300 ml of gradient contained 5.9 log of virus. The cushion layer (150 ml) contained 2.0 log of virus.

TABLE 3. *Per cent recovery of canine distemper virus after combined continuous-flow centrifugation and isopycnic banding in the B-XVI rotor*

Fraction	Sucrose (% w/w)	Log TCID <sub>50</sub>	Total virus originally present (%)
Original		4.4	100.00
Effluent		2.3	8.00
1	5-32	2.0	0.01
2	32-48	5.9	92.00
3	48-50	2.0	0.01

The amount of virus removed from the original tissue culture medium is 100%, and 92% of the total virus is isopycnicly banded at 32 to 48% sucrose. Viral harvest (20 liters) was passed over each gradient in this experiment. The average protein decrease was from 12.6 to 3.2 mg/ml. Therefore, the increase in purity relative to protein concentration was 99.6%.

Immunodiffusion of the purified and nonpurified CDV is shown in Fig. 4a and 4b.

## DISCUSSION

The advantages of continuous-flow zonal rotors for the purification of virus for vaccine and research were discussed by Anderson (1, 2). Among these advantages are the ability of the rotor to concentrate virus from large fluid volumes and separation of the virus without pelleting, which may inactivate some viruses.

In addition, Peck reported that, in the case of influenza, ultracentrifugation of the virus reduced the usual reactions of severe swelling, redness, and pain from 11 to 0.9% of the subjects (10). A further advantage was described by Reimer et al. who reported that influenza virus purified by zonal centrifugation had an enhanced potency in mice (12).

The present study provides a method for preparing vaccines containing CDV and ICH virus of greatly improved purity and perhaps potency. The increase in purity is indicated by separation of the contaminating proteins of the tissue culture medium from the viruses. At present it is not known to what extent this

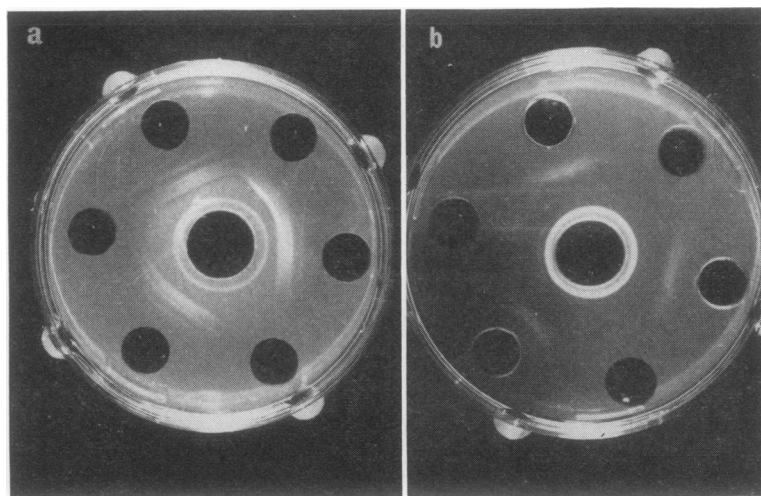


FIG. 4. Comparison of nonpurified and purified CDV by immunodiffusion. (a) Nonpurified virus in alternate outer wells. (b) Purified virus in alternate outer wells. In each case the center well contains rabbit serum against nonpurified virus.

marked increase in purity will affect such factors as the stability or efficacy of the virus. However, the zonal centrifuged vaccine represents a considerable increase in purity which, as Overman and others have pointed out, is essential to development of safer vaccines (9).

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