RETROVIRUS (T)

TUMOR VIRUS PURIFICATION USING ZONAL ROTORS

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SUMMARY

The B- and K-type zonal centrifuge rotors have been used in the author's laboratory for the purification of several RNA and DNA tumor viruses. The viruses have been purified from a variety of sources, including cell culture fluids, cells, tumor tissue, and infected blood plasma. A double density gradient procedure utilizing sucrose gradients has resulted in purified virus concentrates for a series of RNA tumor viruses propagated in cell culture. The DNA herpesviruses implicated in tumorigenesis are cell-associated and are more difficult to purify by density gradient centrifugation alone. However, when applied to the herpesvirus of the Lucké frog kidney tumor and the Epstein-Barr virus in cultured human lymphocytic cells, zonal centrifugation has yielded concentrated virus suitable for biochemical and immunological studies.

The published literature by other investigators employing zonal centrifugation for tumor virus purification is reviewed with respect to the variety of gradient materials and centrifugation conditions used in the different studies.

The development of the zonal centrifuge by Dr. N. Anderson and his associates at the Oak Ridge National Laboratory was supported in part by the U.S. National Cancer Institute. Therefore, it is not suprising that one of the early applications of large-volume zonal centrifugation has been in the field of cancer virus research. The use of zonal centrifuges for cancer virus purification has continued and expanded as significant advances in understanding the mechanism of action and structure of these viruses and the discovery of new viruses have led to increasing demands for purified viruses for research.

For those of you that are unfamiliar with tumor viruses, Table 1 shows the range of viruses under study in this field. Both the RNA and DNA type viruses are under intensive investigation and we will discuss the application of zonal centrifugation to the concentration and purification of both virus classes. There are significant differences between the RNA and DNA tumor viruses that can be important factors in purification results using zonal centrifugation. In general, the RNA tumor viruses are non-cytopathogenic and are released from productively infected cells into the surrounding medium by a budding process from the cell membrane. In contrast, the DNA viruses are intracellular or are released from infected cells by cell degeneration. The most interesting DNA tumor viruses are herpesviruses, which have buoyant densities overlapping those of certain particulate cell constituents released when the infected cells are disrupted or degenerate. These viruses are especially difficult to purify by density gradient centrifugation alone.

The RNA tumor viruses also have buoyant densities that coincide with those of certain cellular constituents. Therefore, if the cell cultures used for virus propagation are not maintained at maximum viability, purification problems can also be encountered with these viruses in relation to contaminating cell microsomal and membrane fragments.

The ideal virus fluid for zonal centrifugation would have the following properties :

- High virus concentration.
- Low level of cellular contaminants
  Best : Non-cytopathogenic budding virus.
  Medium : Highly cytopathogenic virus,
  Worst : Intracellular virus or solid tissue.
- Virus density $>$ 1.20 ($<$ 1.28) to place the virus beyond the banding densities of most cellular membrane, microsomal and mitochondrial components, and yet allow the use of inexpensive sucrose gradients.
- Large virus size (high S value) for high continuous-flow rates.
- Virus not inactivated by gradient.
- Accurate, rapid assay methods.

As we all know, the ideal is rarely encountered, least of all in tumor virus research where one is often called upon to fractionate crude materials containing viruses present in low concentrations. Tissue culture systems are the principal sources of tumor viruses for purification. There are cases, such as the herpesvirus of the Lucké frog kidney tumor, where cell culture systems have not been developed and the tumor tissue itself is the only virus source. Other important sources of tumor viruses are serum or plasma from infected animals (e.g., AMV from chicken plasma), and infected milk in the case of the mouse mammary tumor virus. In general, cell culture systems for the propagation of tumor viruses have the following advantages :

- Readily expandable volume
- Control of contaminating viruses
- Can switch to different viruses quickly
- Less cost per volume (not necessarily less cost per virus).

In contrast, viremic animal fluids and tissues generally contain :

- Higher virus concentrations
- Higher biological activity per virus particle
- Less cellular contamination (serum & plasma).

TABLE 1

KNOWN TUMOR VIRUSES (9/72)

RNA

Type C
Leukemia and sarcoma
Chicken, snake, mouse, rat, hamster, cat, human (?
Density 1.15-1.18, diam. 100 nm.

Type B
Mammary tumor
Mouse, monkey, human (?)
Density 1.16-1.18, diam. 110 nm.

DNA

Herpesviruses
Lymphoma, carcinoma
Chicken, frog, monkey, human (?)
Density 1.19-1.21 (mature), diam. 150 nm.

Papovaviruses (polyoma, SV40, papilloma)
Sarcomas
Mouse, hamster, rabbit
Density > 1.25, diam. 45-55 nm.

TABLE 2

Typical RNA Tumor Virus Fluid

Virus concentration : 1 - 5 × 10^8 per ml.
Growth medium : EMEM with 5 % fetal calf serum.
Total protein : 5 mg per ml.
pH 7.0-7.4
Clarified at harvest at 4000 g × 10 min.
May be frozen at below - 60°C.

TABLE 3

Assays for RNA Tumor Viruses

Physical
- Electron Microscopy (neg. stain & thin sect.)
  - Virus count
  - Morphology
  - Purity

Biochemical
- Reverse transcriptase enzyme
- 60-70S RNA, total RNA
- Total protein
- Gel analyses of viral & host proteins & nucleic acids.

Immunological
- Gel diffusion
- Complement fixation
- Immunofluorescence

} Specific reagents for envelope and internal antigens (gs & enz.)

Biological
- Infectivity *in vivo*
- Infectivity *in vitro*

Let us consider first the purification of the RNA tumor viruses. In Table 2 are listed the properties of a typical RNA tumor virus tissue culture fluid as obtained for such well-defined systems as the type C murine and feline leukemia viruses.

The assay methods for the RNA tumor viruses include a battery of physical, biochemical and biological tests for concentration, activity and purity as shown in Table 3. The purification methods, degree of purification and assay methods used to measure progress are often dictated by the end-uses of the final virus concentrate. For example, if the purified virus concentrate is to be used as an immunological reagent in gel diffusion tests, these gel diffusion tests should be employed where practical to monitor the purification process.

RNA tumor viruses purified by sucrose gradient centrifugation have proven to be satisfactory reagents for a variety of structural, immunological and biochemical studies (1). In our laboratories, we have employed a double sucrose gradient procedure using zonal rotors as outlined in Figure 1.

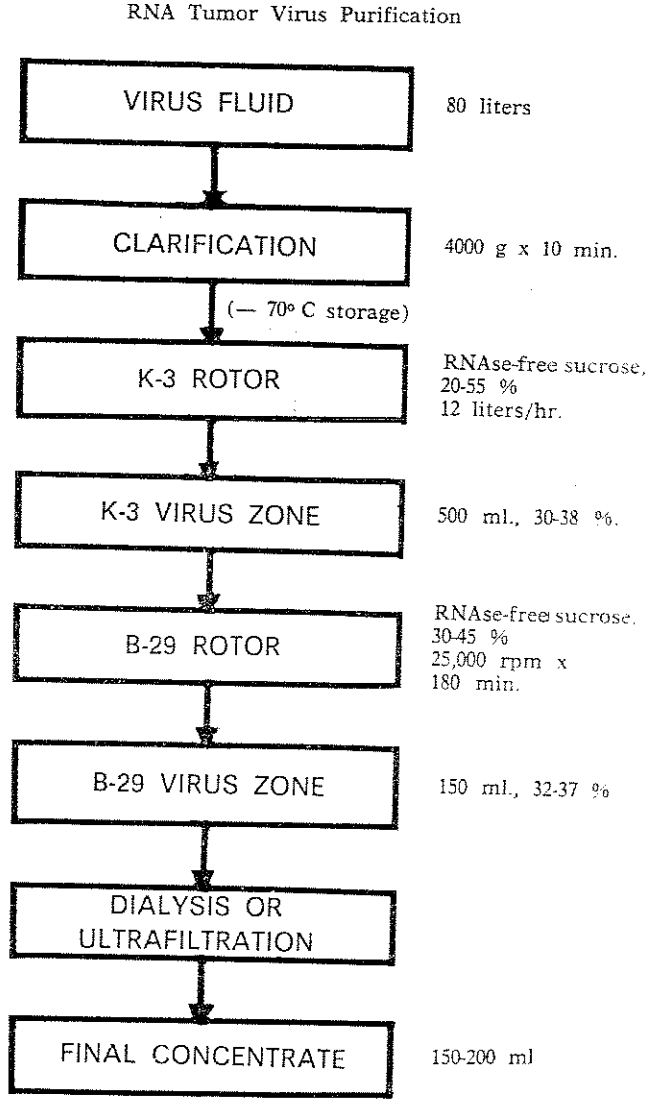


Fig. 1. - *Flow chart for purification of RNA tumor viruses by double sucrose gradient zonal centrifugation.*

The primary recovery employs the Model K zonal centrifuge (Electro-Nucleonics) with the K-3 continuous-flow rotor, capacity 3,2 liters. Figure 2 is a schematic presentation of the equipment layout for K-3 rotor operation. If sufficient volumes of virus fluids are produced to warrant K Centrifuge runs every 48 hours or sooner, the virus fluids are held at 0-4°C without freezing or batch clarification, and the K-6 rotor with built-in clarifier is employed in place of the K-3 rotor. The feed and receiver vessels are 20-liter polypropylene jugs each fitted with a stainless steel dip tube and bacteriological air filter passing through the rubber-stopper cap. Where possible, connections between equipment are made using autoclavable rubber or plastic tubing and stainless Luer-Lok connectors. The feed bottles connect to a common manifold line leading to a peristaltic tubing pump (Sigmamotor TM35, 50-500 ml per min). This, in turn, leads to a four-way valve which allows rapid switching of the feed to either the top or bottom of the rotor. The feed and receiver jugs are sterilized by autoclaving, but the K-3 rotor and attached lines, including the flow cell for the recording UV absorbance monitor, are sterilized in our laboratories by filling the system with 70 % isopropanol and allowing a 6-12 hours contact period.

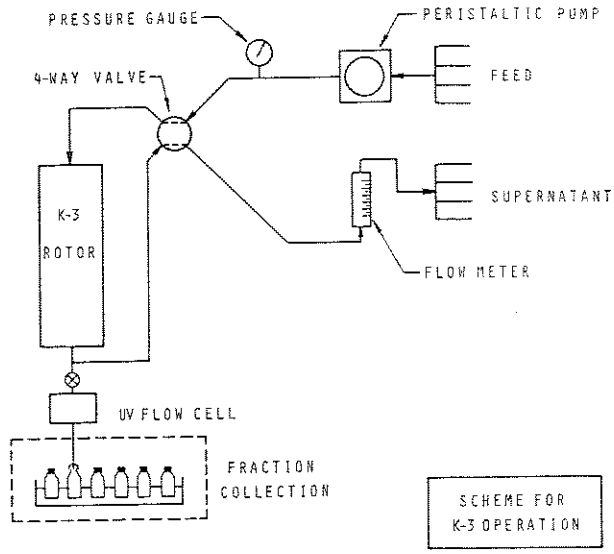


Fig. 2. - *Schematic arrangement of equipment for operation of Model K centrifuge with K-3 continuous-flow rotor.*

To start a run, the vacuum and cooling systems are activated, the alcohol is displaced from the rotor and lines by sterile air, and the system is flushed with cold, sterile, deionized water. The rotor is then filled with cold sterile buffer, in our laboratories usually 0,1*M* NaCl, 0.01*M* Tris, and 1mM EDTA, pH 7.2-7.4. The sucrose gradient is introduced to the bottom of the rotor using a separate peristaltic pump. For recovery of the RNA tumor viruses from large volumes of fluids by continuous flow centrifugation, we have been using a two-step gradient of 20 % (w/w) and 55-60 % sucrose in buffer. The sucrose is special density gradient material free of detectable RNase activity (Schwarz/Mann). In the two-step gradient, the 20 % sucrose serves as a barrier between the virus zone at 30 %-38 % sucrose and the low molecular weight or slow sedimenting components in the sample zone. The 55-60 % sucrose serves as a cushion between the virus zone and rotor wall. A liter of 20 % sucrose and normally 1200 ml of 55-60 % sucrose compose the gradient.

The amount of 55-60 % sucrose will vary with the sample volume. We generally process 60-80 liters per run, but continuous-flow runs have been made with as much as 150 liters.

During the time period of the run, diffusion gives an approximately linear gradient between the sample zone and the heavy sucrose cushion. For smaller sample volumes (5-30 liters), a preformed linear gradient of 2 to 3 liters is introduced into the rotor using a gradient pump.

The rotor is accelerated to operating speed, generally 35,000 rpm (83,000 g, max.). During acceleration, buffer is pumped through the rotor to maintain the sample zone clear of sucrose. Sample flow is initiated at operating speed and maintained at about 12 liters per hour. Flow rates higher than 12 liters per hour result in significant (> 5 %) losses of the RNA tumor virus in the supernatant medium. At the end of the sample flow, the rotor is held at operating speed for one hour banding time to allow the virus that entered the gradient at the end of the sample flow to reach the vicinity of the main virus zone.

The rotor is then decelerated to rest, and sterile air is pumped to the rotor top to displace the gradient from the rotor bottom at approximately 50 ml per minute. The gradient runs through the flow cell of the recording UV absorbance monitor (ISCO) and is collected in 100 ml fractions in sterile disposable 4 oz. screw-cap prescription bottles. In lieu of a biologically isolated refrigerated fraction collector, we place the empty sterile fraction bottles in a tray of crushed ice plus disinfectant and freeze the tray at —20°C for several hours. For biocontainment, the frozen tray of bottles is placed in an inflatable polyethylene glove bag (I^2 R) with sterile plastic tubing leading through the wall of the bag and a bell dispenser at the end of the tubing inside the bag. After collecting the desired number of fractions on a low cart close to the bottom of the centrifuge, the line leading into the bag is flushed with disinfectant (Wescodyne, 1 %) and clamped. The bag is disconnected from the centrifuge line and the closed fraction bottles are wiped with disinfectant and removed from the bag in a biohazard hood.

A K-10 rotor (8-liter capacity) is occasionally employed on a batch basis for the recovery of virus from 2 to 7 liters of fluid. The rotor loaded at rest with the sample and two-step sucrose gradient, accelerated to 35,000 and held for 120 to 180 min. Unloading and fraction collection are carried out in the same manner as described for the K-3 rotor.

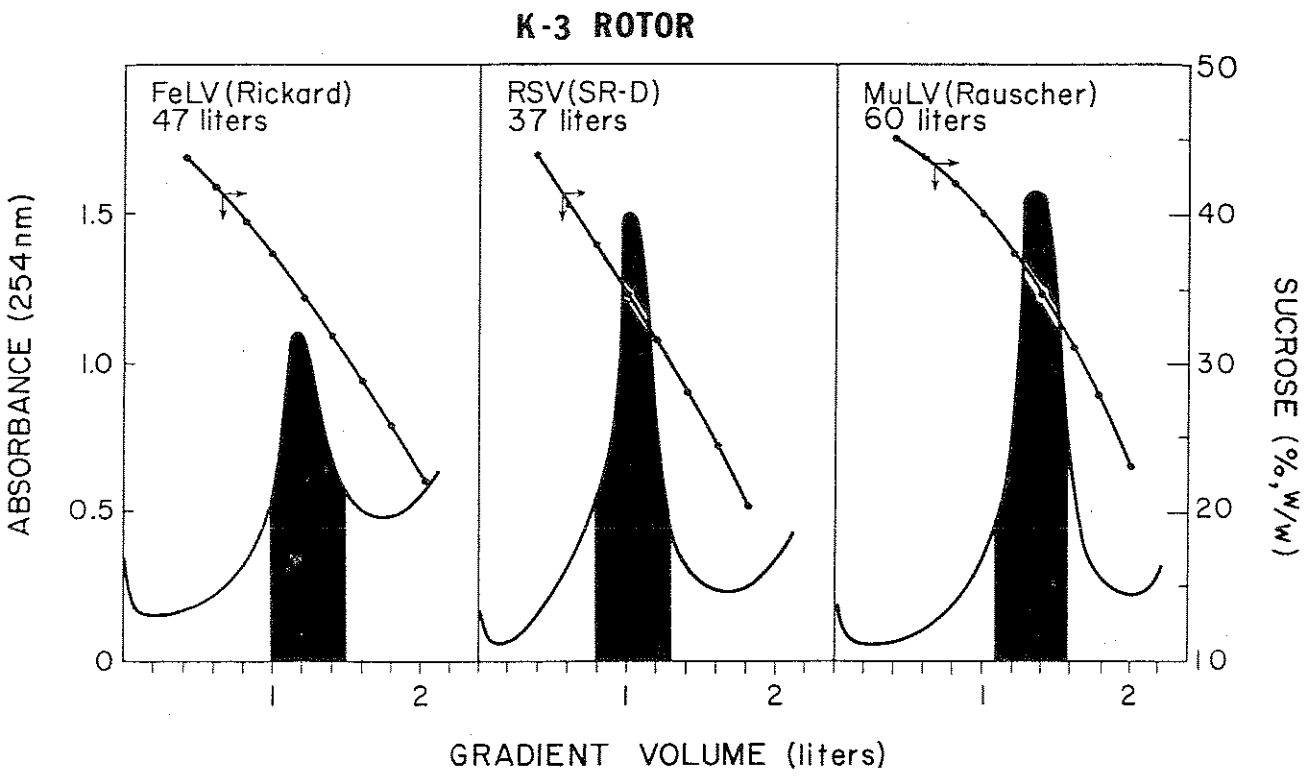


Fig. 3. - *Gradient profiles from continuous-flow recovery of various RNA tumor virus fluids in a K-3 rotor at 12 liters/hr, 35000 rpm. The shaded areas show the virus zones used for further purification in a B-29 rotor. (Reprinted from ref. 1 with permission of the publisher).*

Figure 3 shows the UV profiles at 254 nm from several K centrifuge runs with various RNA tumor viruses. The virus band at 30-38 % sucrose, generally five 100 ml fractions, is further purified and concentrated by a second sucrose gradient centrifugation using the same capacity as the B-29 or B-15 zonal rotors. The B-29 rotor has approximately the same capacity as the B-15 rotor, but

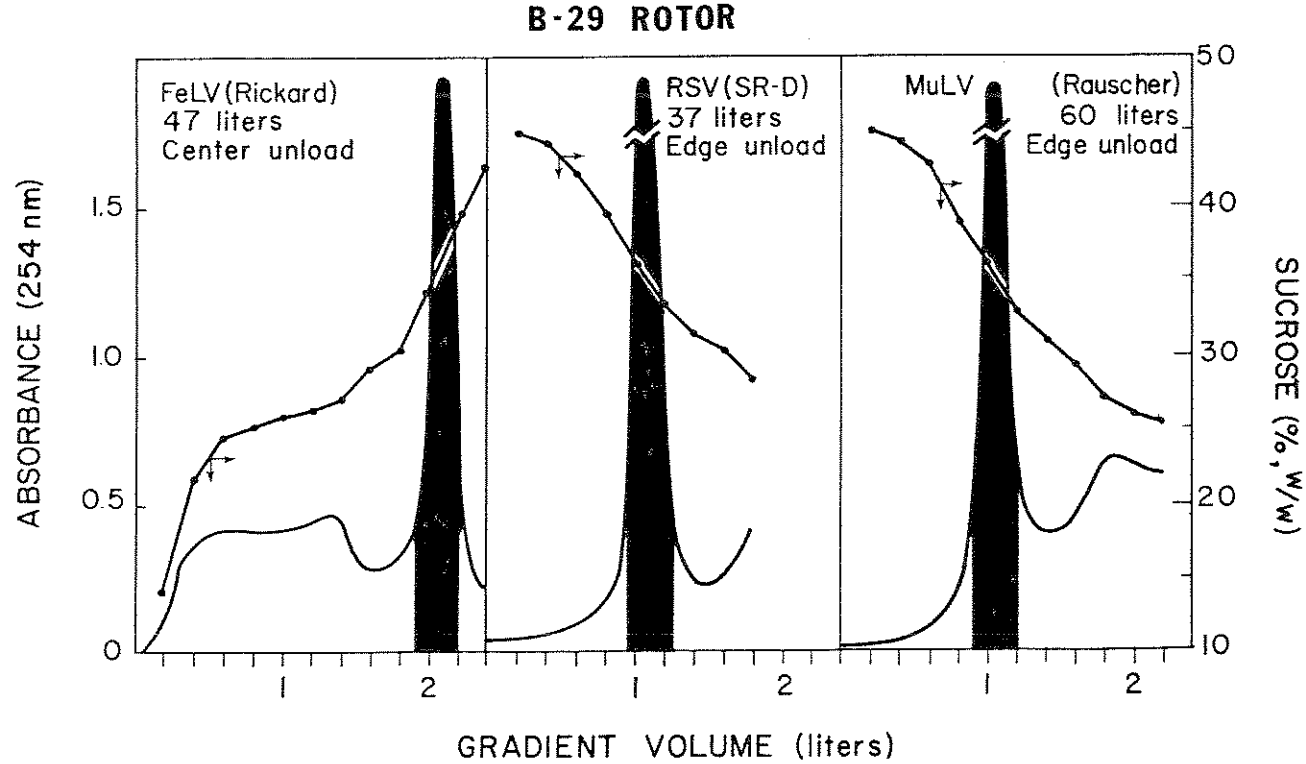


Fig. 4. - *Gradient profiles from second sucrose gradient centrifugation of RNA tumor viruses in a B-29 rotor using virus zones from K-3 rotors shown in Fig. 3 as starting samples. Centrifugation was at 25000 rpm for 180 min with a 30-45 % (w/w) sucrose gradient. Starting samples were diluted to below 30 % sucrose with buffer before loading B-29 rotors. Shaded areas show the virus zones taken for the final concentrates. (Reprinted from ref. 1 with permission of the publisher).*

its design allows loading and unloading at the rotor edge or center with equal resolution, a feature that gives flexibility and speeds up the handling time. The virus zone from the K gradient is diluted with sterile buffer to below 30 % sucrose and is followed into the rotor by either a preformed linear 750 ml gradient ranging from 30 to 45 % sucrose, or a two-step gradient consisting of 350 ml of 30 % sucrose and 400 ml 45 % sucrose. Centrifugation is at 25,000 rpm for 3 hours.

Figure 4 shows the UV profiles at 254 nm for the gradients from the B-29 rotor for the viruses shown in Figure 3. From 40 to 60 liters, we have reduced the virus volume to about 150 ml and significantly increased the purity. The final virus concentrates are freed of sucrose by dialysis, gel filtration, membrane ultrafiltration, or centrifugation to a pellet and resuspension in the desired buffer. We adjust the virus level to 2×10^{11} particles per ml using rapid quantitative negative stain electron microscopy to measure the concentration (2). Such concentrates have the properties shown in Table 4. Comparing the ratios of virus concentration to protein for the final virus concentrates after two zonal centrifugations and the starting cell culture fluids, we find approximately a 4000-fold increase in virus purity.

TABLE 4

Purified RNA Tumor Virus Concentrates

Virus concentration	2×10^{11} particles/ml
Total protein	0.4-0.5 mg/ml
CF titer	1 : 128-1 : 256 vs. 4 antibody units
Reverse transcriptase	
Endogenous template	> 0.5 pmol ^{3}H-TTP incorp. per 30 min. per 0.05 ml. virus with « std. » mixture of Scolnick et al. (3)
Exogenous template (poly rC : oligo dG)	> 100 pmol ^{3}H-TTP incorp.

Many of these concentrates show little or no detectable DNA or ribosomal or other cellular RNA by sentitive analytical gel electrophoresis techniques (J. Bader, personal communication). Preparations of this type have been used for recent structural studies of Rauscher murine leukemia virus (4) and the isolation of viral subunits (5). Electron microscopy also serves as a method for evaluating the purity and morphology of the RNA tumor virus preparations. Figure 5 is a thin-section electron micrograph of a pellet from an unprocessed tissue culture fluid containing Rauscher murine leukemia virus; Figure 6 is a thin-section from a purified virus preparation that has been processed through the double sucrose gradient procedure; and Figure 7 is a negative stain of the purified virus. Note the obvious increase in virus purity.

The most prominent herpesvirus in tumor virus research is the Epstein-Barr virus (6), or EBV, found in cell cultures derived from Burkitt's lymphoma and in certain lymphocytic cultures from apparently normal individuals. The yield of virus in these cultures is variable, but even under the most favorable conditions for virus replication, only 5-10 % of the cells are infected and extracellular virus concentrations reach only about 10^7 particles per ml, 10-fold less than with the RNA tumor viruses. An additional problem is that there are three morphological forms of the EBV present in most cultures, reflecting the abortive nature of the EBV infection. These forms are the mature herpesvirus with inner nucleoid and outer envelope (rarest form), the unenveloped nucleated virus, and the unenveloped empty capsids which normally are the most prevalent form. The densities of these forms range from 1.18 for the empty capsids to 1.26 for the unenveloped nucleated particles, with the mature enveloped virus banding at the intermediate density of 1.20. These higher densities compared to the RNA tumor viruses allow somewhat higher flow rates during continuous-flow zonal centrifugation using the K-3 rotor. We use flow rates of 15 liters per hour for EBV recovery and batch volumes of 80-150 liters.

The two-step sucrose gradient for EBV work consists of 20 % and 60 % sucrose solutions. Typically, each 100 ml fraction from the K centrifuge gradient is reduced to 5 ml by membrane ultrafiltration or pellet centrifugation and resuspension. The concentrates are examined by negative stain electron microscopy for the distribution of virus concentration and morphological form before proceeding to further purification.

The intracellular virus also is recovered from the infected cells by sucrose gradient zonal centrifugation (7). The cell packs are suspended in hypotonic buffer and disrupted by sonication or homogenization. We find that fluorocarbon extraction of the cell homogenates is an effective method of separating the virus from the majority of nonviral protein prior to zonal centrifugation. This technique was first described in 1956 by Gessler et al. (8). After vigorous extraction of the cell homogenates with an equal volume of cold trichlorotrifluorethane and moderate centrifugation to separate the phases, the virus is found in the top aqueous phase whereas the protein accumulates as a gel at the interphase. We normally carry out two extractions of each homogenate. The aqueous phase is then fractionated on a 10 to 60 % sucrose gradient in a B-15 or B-29 rotor. The preliminary fluorocarbon extraction allows us to place the virus from 20-25 gms of packed cells on a one-liter gradient. Without this reduction in protein, the gradient would be limited in capacity to about 8 grams of cell pack homogenate.

Some results with EBV purification are shown in Figures 8 and 9 and show that high-titer concentrates can be obtained from this system for biochemical and immunological studies.

Another interesting herpesvirus implicated in tumorigenesis is that found in the Lucké frog kidney tumor (9). In our laboratory, tumors rich in virus particles were homogenized or sonicated and then separated in nuclear, mitochondrial supernatant fractions prior to sucrose zonal centrifugation (10, 11). Figures 10 and 11 present some of the results of the experiments. Gradient fractions rich in an enveloped, nucleated form of the herpesvirus from certain tumors have induced renal tumors when injected into developing frog embryos (12).

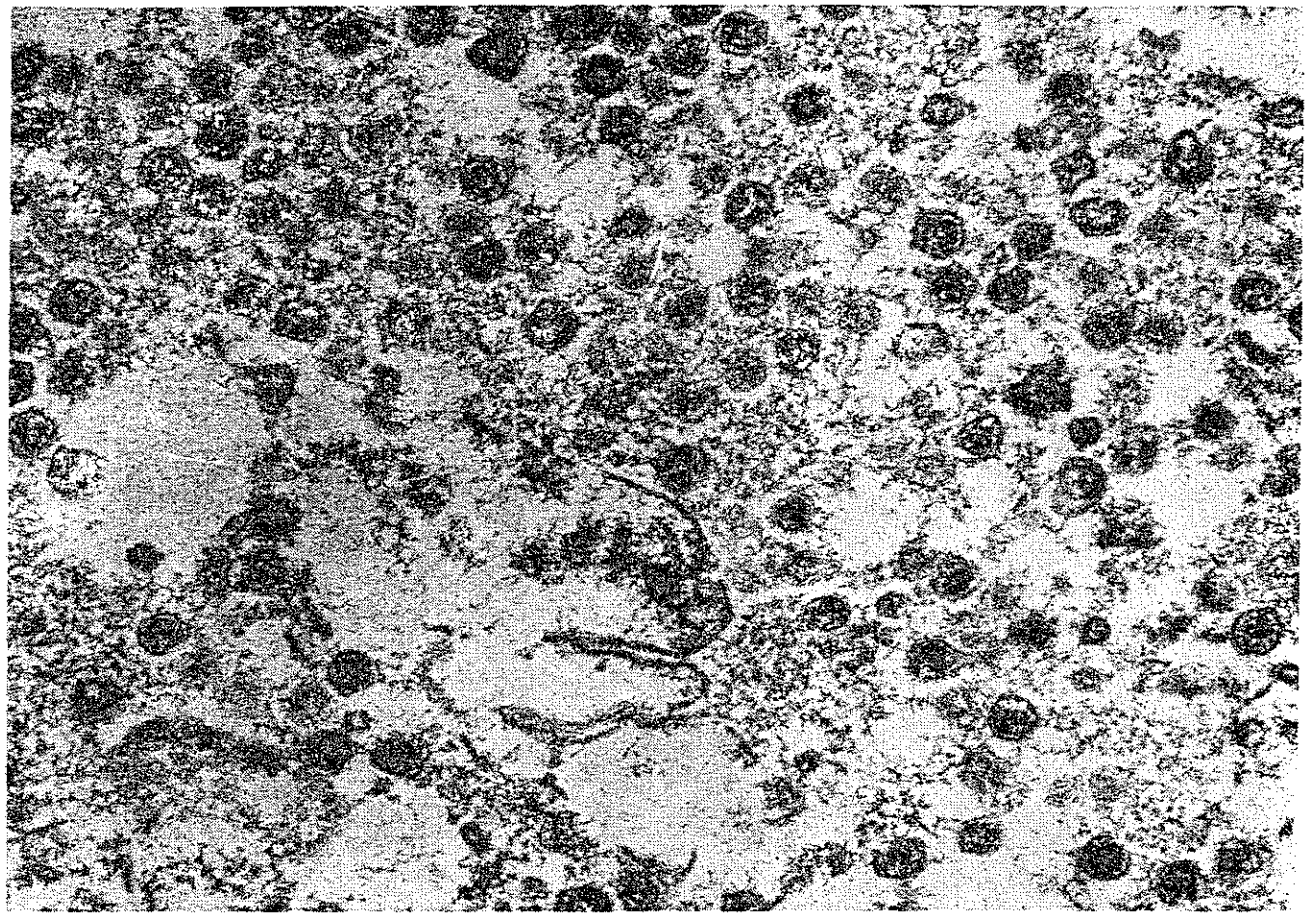


Fig. 5. - *Electron micrograph of unprocessed clarified cell culture fluid containing Rauscher murine leukemia virus. Fluid was centrifuged at 6000xg for 30 min and resulting pellet was thin-sectioned (magnification 76,000X).*

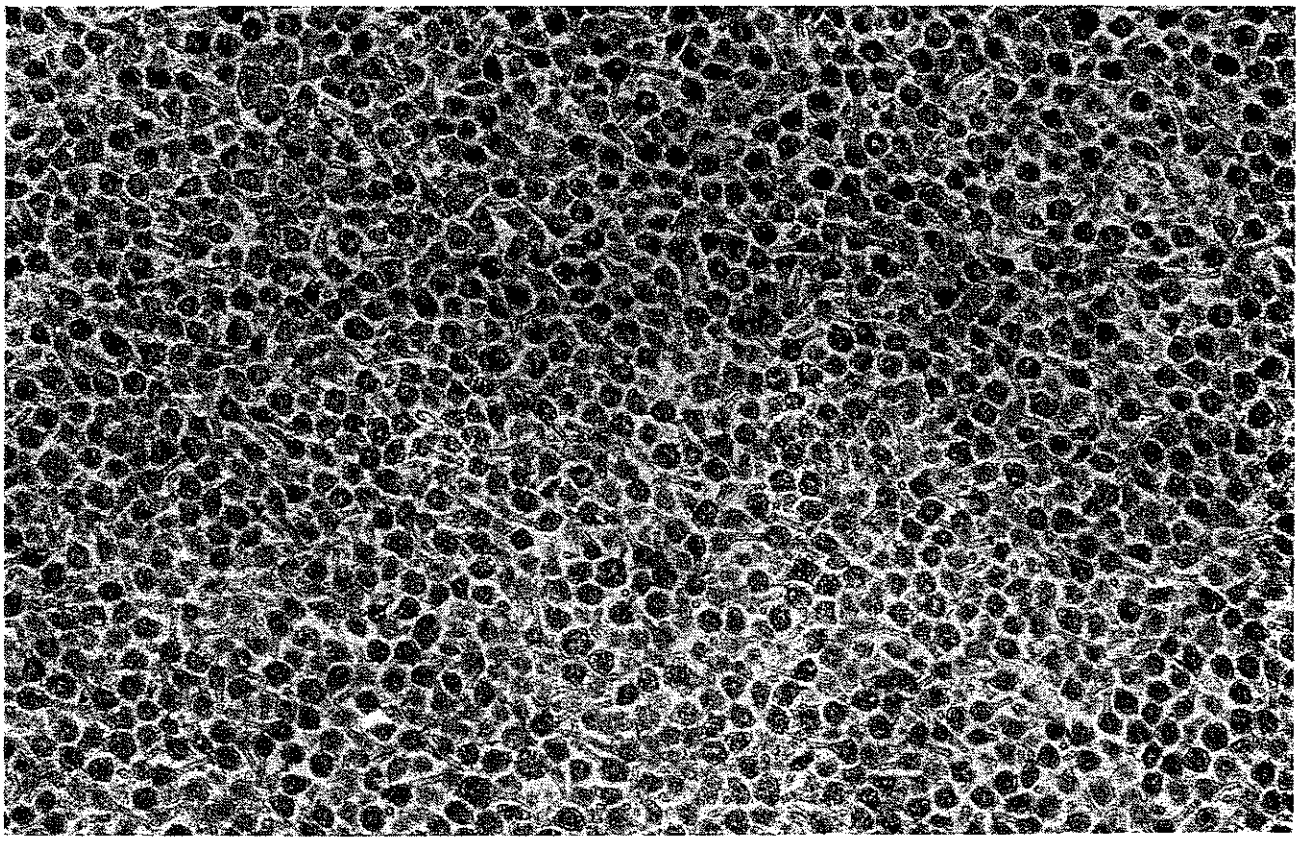


Fig. 6. - *Electron micrograph (thin-section) of Rauscher murine leukemia virus from cell culture fluid after double sucrose zonal centrifugation (magnification 40,000X).*

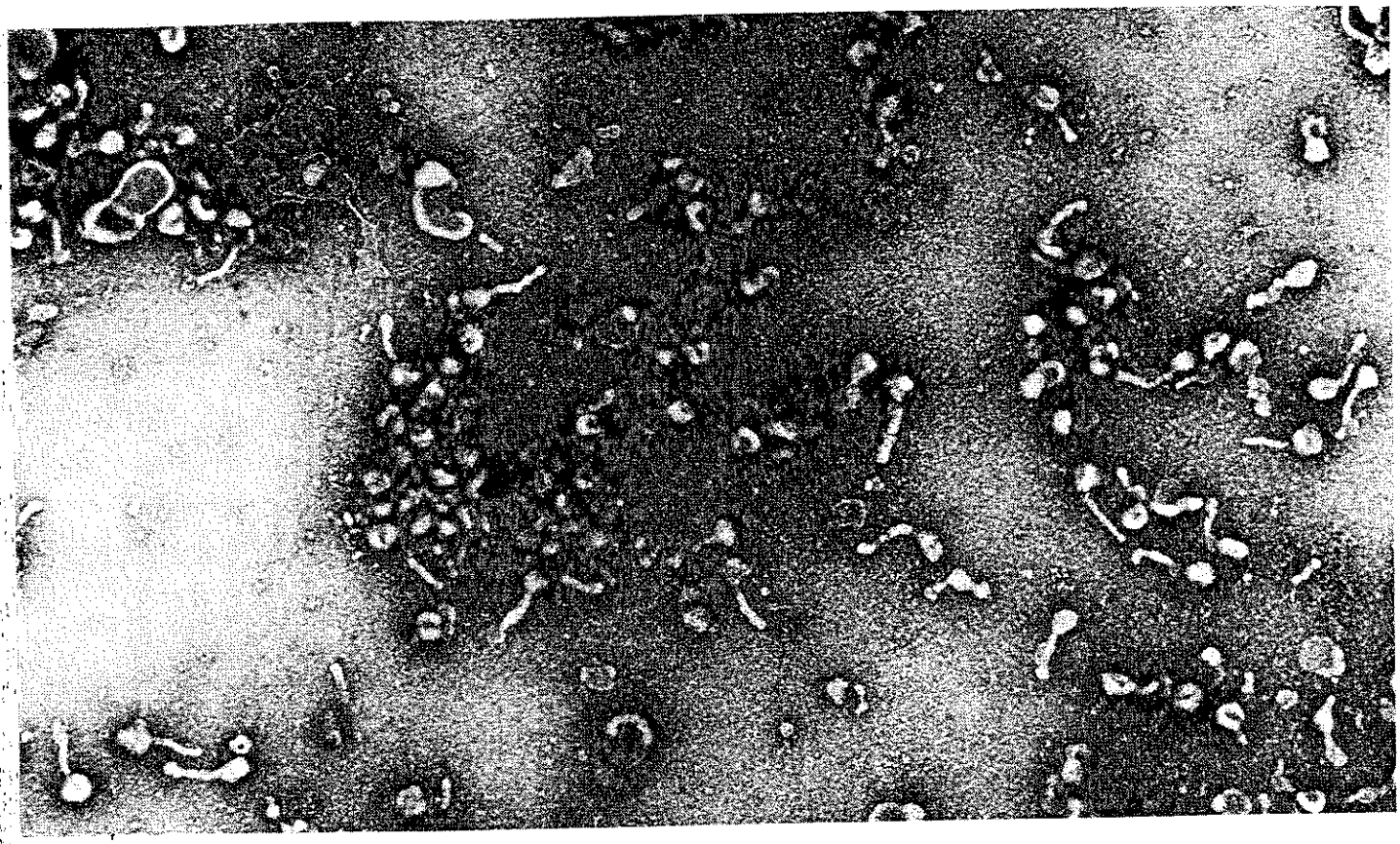


Fig. 7. - *Electron micrograph (negative-stain) of same virus preparation seen in Fig. 6 (magnification 40,000X).*

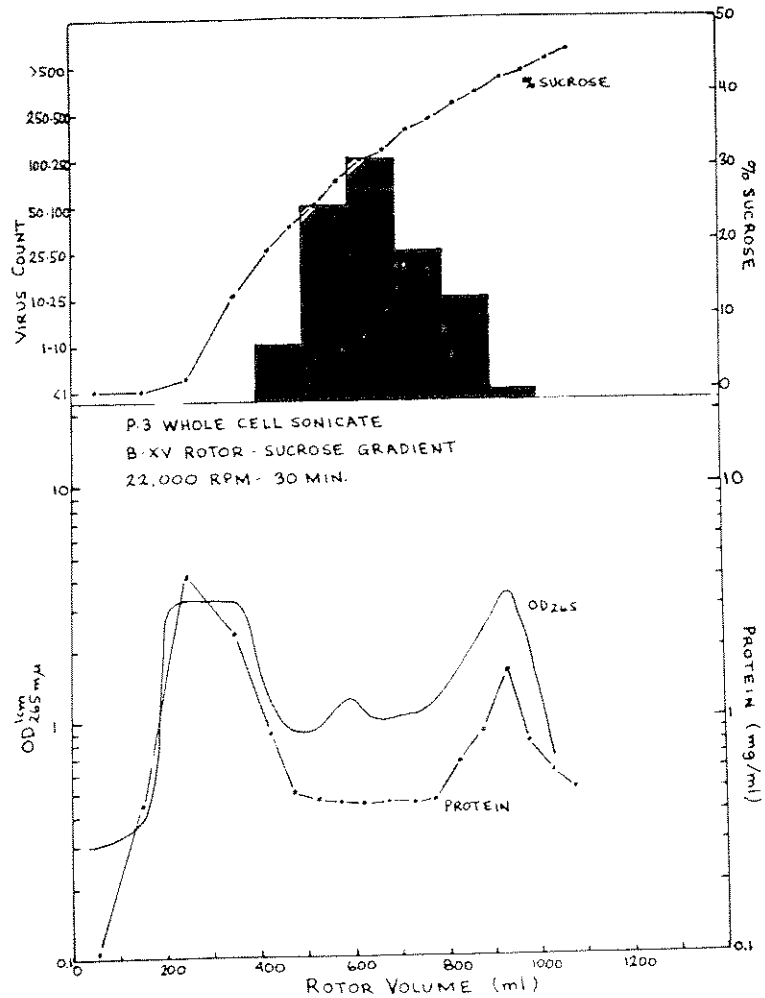


Fig. 8 a.

Fig. 9. - *Electron micrograph (negative-stain) of Epstein-Barr herpesvirus recovered from cell culture supernatant fluid by zonal centrifugation (magnification 50,000X).*

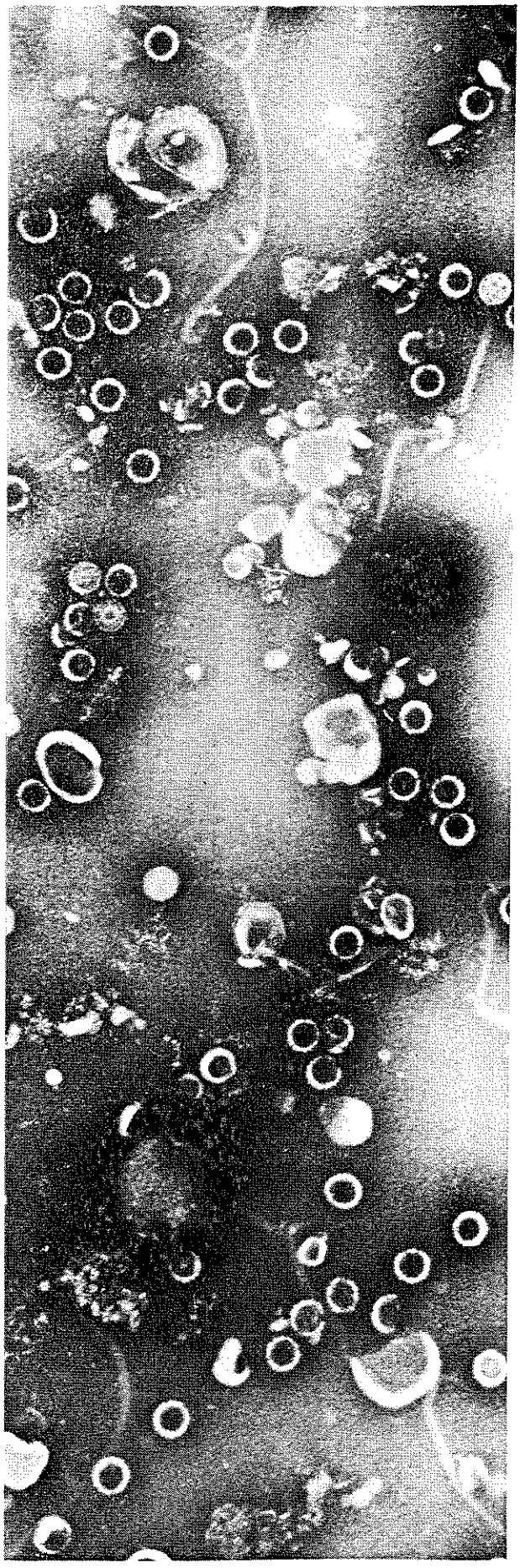
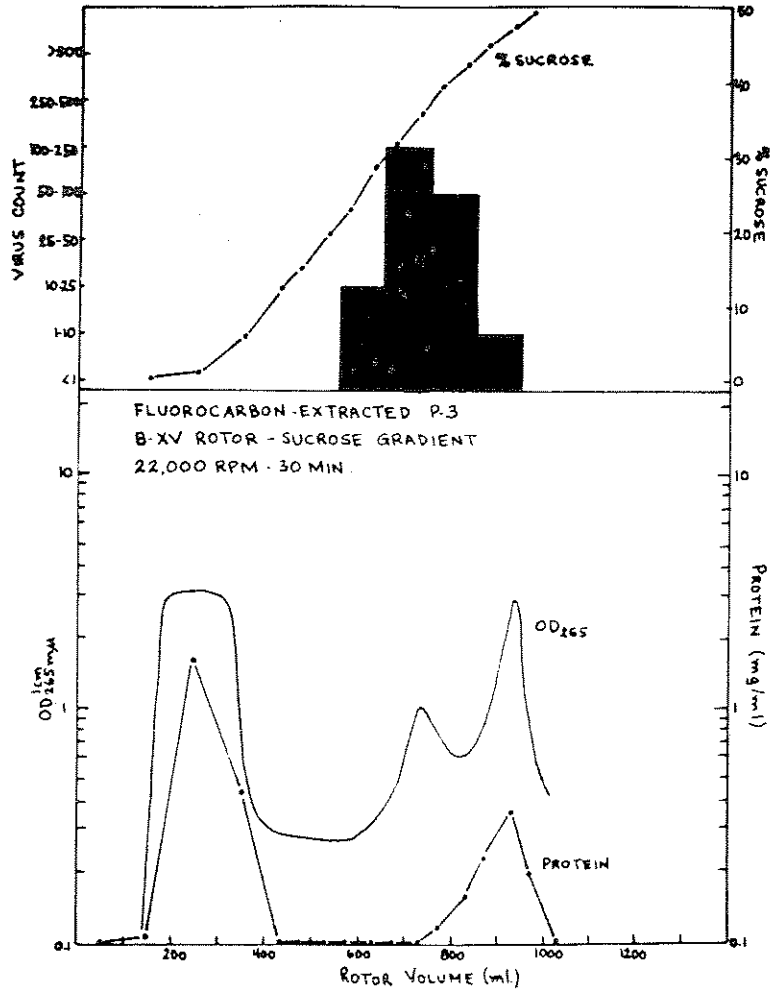


Fig. 8. - *Gradient profiles from zonal centrifugation of Epstein-Barr herpesvirus : (a) 8 ml cell pack disrupted by sonication on a 1-liter 10-60 % (w/w) sucrose gradient; (b) 8 ml cell pack disrupted by sonication and fluorocarbon extracted prior to zonal centrifugation. Note the reduction in protein throughout the gradient. Virus concentrations on 5-fold concentrates of each 50 ml fraction expressed as virus particles per grid square where one virus/sq equals 5 × 10^7 particles/ml. (Reprinted from ref. 7 with permission of the publisher).*



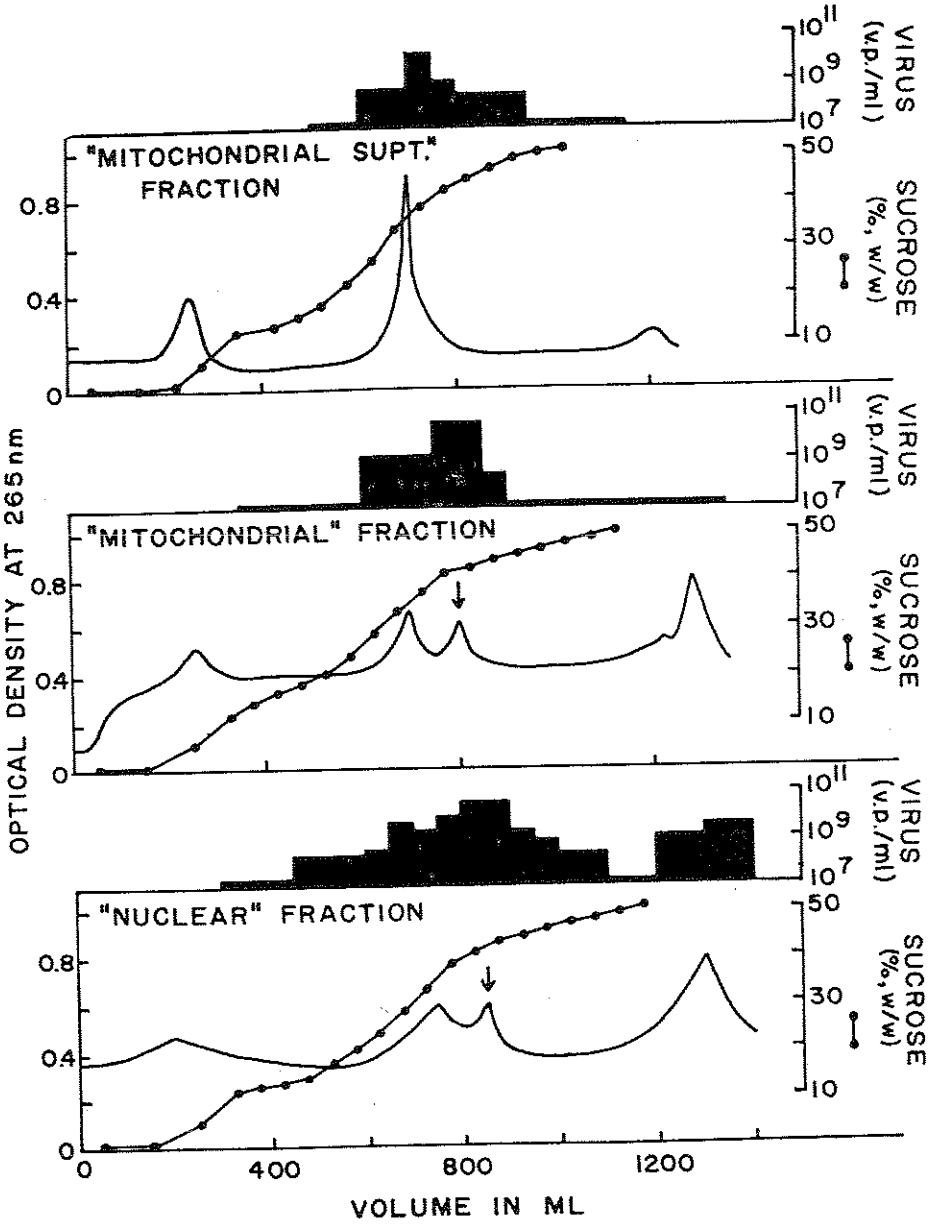
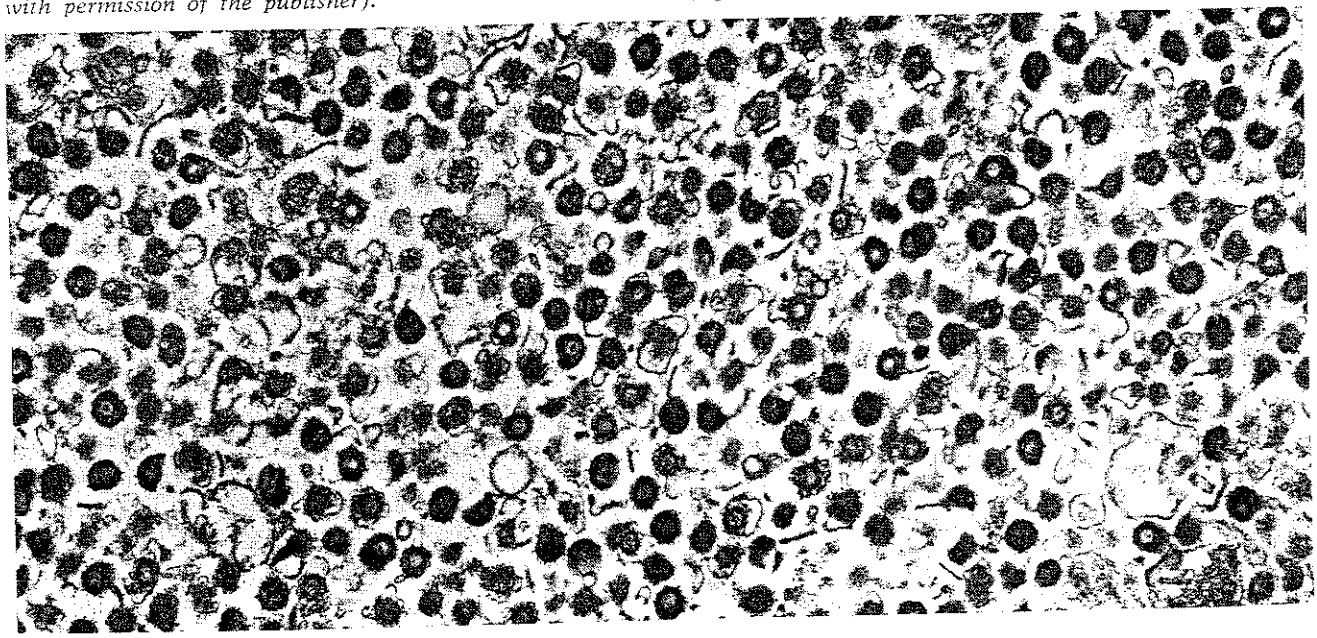


Fig. 10. - *Rate-zonal centrifugation of various tumor fractions from Lucké frog kidney tumor. Centrifugation was at 25,000 rpm for 60 min in the B-XV rotor with a 1-liter 10-60 % (w/w) sucrose gradient. (Reprinted from ref. 11 with permission of the publisher).*

Fig. 11. - *Electron micrograph (thin-section) of herpesvirus isolated from Lucké frog kidney tumor by zonal centrifugation (magnification 40,000X).*



One is not limited to sucrose gradients in tumor virus purification. We have worked successfully with other gradient solutions such as potassium citrate and potassium tartrate for the RNA and DNA tumor viruses, and glycerol, Ficoll (Pharmacia), and Ficoll-heavy water gradients for the RNA tumor viruses.

Table 5 summarizes the published literature from other laboratories on the application of zonal centrifugation to the purification of tumor viruses. We apologize for any inadvertent omissions.

TABLE 5

PUBLISHED LITERATURE ON TUMOR VIRUS PURIFICATION USING ZONAL ROTORS

VIRUS	SOURCE	GRADIENT	ZONAL ROTOR	CENTRIFUGATION	REF.
Mouse leukemia	Cell culture; mouse plasma	Sucrose; pot. citrate; pot. tartrate	B-IV; B-IX	B-IV, 21-28000 rpm × 60-120 min	13
				B-IX, 2-3 liters/hr at 28-32000 rpm	4
Polyoma	Cell culture	Sucrose	B-IV	40000 rpm × 60 min	14
Mouse mammary tumor	Mouse milk	Sucrose	B-XIV	27000 rpm × 40 min	15
Epstein-Barr	Cell culture	Sucrose	B-XIV	35000 rpm × 30 min	16
Feline leukemia	Cell culture	Sucrose	B-IX	1.8-2 liters/hr at 35000 rpm	17
Mouse leukemia	Cow's milk seeded with mouse plasma; mouse spleen extracts	Sucrose	B-XVI	3-6 liters/hr at 38000 rpm	18

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