

## Adaptation and Infection of Mouse Bone Marrow (JLS-V9) Cells in Suspension Culture for Production of Rauscher Leukemia Virus

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JLS-V9 mouse bone marrow cells were readily adapted to suspension culture, chronically infected with Rauscher leukemia virus (RLV), and subsequently grown in 7.5- and 14-liter New Brunswick fermentors. The suspension-type cell system can be modified to produce virus with clearly defined properties, such as high ribonucleic acid-dependent deoxyribonucleic acid polymerase (RDDP) activity, high particle count, and high infectious particle count. Biological and biophysical properties of suspension-produced RLV were not affected by concentration and purification employing continuous-flow and rate-zonal centrifugation procedures. The RDDP assay was standardized and showed a linear incorporation of  $^3\text{H}$ -thymidine 5'-monophosphate ( $^3\text{H}$ -TMP) up to 30 min. Further characterization indicated that a high percentage of  $^3\text{H}$ -TMP incorporation was due to RDDP.

There is an increasing demand for high purified Rauscher leukemia virus (RLV) and its subviral components in the investigation of the relationship between oncornaviruses and malignant disease. To satisfactorily meet this demand, methods of improving virus yields from chronically infected cell lines releasing C-type particles should be investigated, developed, and implemented.

In 1972 Syrewicz et al. (10) reported a method for production of murine ribonucleic acid (RNA) tumor viruses and C-type virus particles in relatively large quantities in roller bottle culture. Recently Smith and Bernstein (9) described procedures for production of Rous sarcoma virus (RSV) in chicken embryo fibroblast (CEF) in roller bottles. The RSV-transformed CEF did not grow in Spinner flask cultures. Large-scale suspended cell production of Moloney leukemia virus in tissue culture using the infected MT 77 cell line was previously reported by Toplin, Riccardo, and Jenson (11). This report represents an additional attempt to propagate a C-type virus particle in a suspension culture system.

The purposes of this investigation were to (i) adapt the JLS-V9 cells previously propagated using monolayer systems (12) to suspension culture, (ii) chronically infect the suspension-adapted cells with RLV, (iii) characterize RLV replication in 7.5- and 14-liter fermentors, and

(iv) evaluate the final virus product after concentration and purification procedures routinely used for roller bottle-produced virus. The replication (particle counts), infectivity of the virus, and levels of deoxyribonucleic acid (DNA) polymerase (DP) activity using endogenous template were also followed during the course of virus replication.

### MATERIALS AND METHODS

**Cell line and virus source.** The cell line (JLS-V9), established from mouse bone marrow, and chronically RLV-infected monolayer JLS-V9 cells (12) used in these studies were obtained from the John L. Smith Laboratory for Cancer Research, Charles Pfizer and Co., Inc., Maywood, N.J.

**Fermentors.** The fermentors (New Brunswick Scientific Co., New Brunswick, N.J.) used were a series of three 14-liter glass fermentors (FS-300). Each unit contained a set of turbine impellers, operated between 100 and 150 rpm. Separate ports were provided for introducing medium and inoculum and for withdrawing samples. A mixture of 95% air and 5%  $\text{CO}_2$  was sparged through a single orifice immediately below the turbine-type impeller. Sparging at a low rate of 100 to 150  $\text{cm}^3/\text{min}$  eliminated the need for antifoam agents and provided the necessary physiological conditions for growth of cells. A working volume of 12 liters was used.

A single 7.5-liter (working volume 6-liter) New Brunswick fermentor was also used in RLV production. The single-unit fermentor was outfitted with two additional ports holding a pH electrode and an

oxidation/reduction potential (ORP) electrode. The electrodes were connected to a recording console for monitoring pH and ORP. All other systems such as agitation, sparging, and temperature were controlled for production of RLV. A 7.5-liter New Brunswick unit was also used to provide inoculum for the 14-liter New Brunswick units.

Cultures were grown in RPMI 1640 medium, supplemented with 0.12% methylcellulose (15 cps), 10% fetal calf serum, 100 U of penicillin per ml and 100  $\mu$ g of streptomycin per ml. In 14-liter units the growth medium was sterilized by filtering directly into each unit using a Seitz plate and frame filter (3). The growth medium was poised by allowing the medium to incubate overnight in the 37 C incubator.

**Infected cell cultivation.** The JLS-V9 chronically infected suspension cell was used for RLV production. The infected cell inocula for the 7.5- and 14-liter New Brunswick units obtained from a frozen seed stock (transfer no. 8) were added to Spinner vessels, then transferred into a 7.5-liter New Brunswick unit and finally into the 3-unit, 14-liter vessels. A flow diagram of this procedure is presented in Table 1.

In steps 1 through 7 the Spinner vessels were operated at an impeller speed of 300 rpm and sparged with 5% CO<sub>2</sub> and 95% air at 10 ml per min per liter of culture. The cell culture volumes were increased to the maximum working capacity of the various size Spinner vessels. Medium was added or the cells were transferred to a larger Spinner vessel when a viable cell count reached  $5 \times 10^5$  to  $10^6$  cells/ml.

**Assay procedures:** (i) **viable cell count.** Cell counts were made by centrifuging 4 ml of cell suspension at  $1,000 \times g$  for 15 min. The supernatant was decanted, and 1.0 ml of 0.25% trypsin was added. The cell preparation was aspirated several times with a syringe, and enough growth medium was added to resuspend the cells to the original 4-ml volume. Viable cells were quantitated by mixing 0.5 ml of 0.5% basal salt solution of Erythrosin B with 1.0 ml of trypsinized cell suspension and counting in a hemocytometer. Total cell counts were likewise obtained.

(ii) **In vivo assay.** The assay employed for determining the in vivo infectivity of RLV produced in suspension culture was patterned after Pulznik and Sacks (8) and Axelrad and Steeves (1). BALB/c mice were intravenously inoculated by means of the caudal vein with 0.50 ml of the appropriate dilution of RLV. Nine days after injection, the mice were sacrificed by use of CO<sub>2</sub> gas, and their spleens were removed and placed in Bouin solution (6). After 24 h of contact with the fixative, foci which had developed on the spleen were macroscopically enumerated and virus titers were expressed as focus-forming units (FFU) per milliliter.

(iii) **Total particle count.** Culture fluids containing RLV were pelleted and resuspended in tris(hydroxymethyl)aminomethane (Tris) buffer to a  $\times 100$  concentration for electron microscopy. Samples were prepared in 2% phosphotungstic acid, pH 4.2, and examined in a Hitachi HU-12 electron microscope at 75 or 100 kV. Virus particle counts (VPC) were done by the negative staining method of Monroe and Brandt (7).

TABLE 1. Build-up of inoculum for production of Rauscher leukemia virus in 14-liter New Brunswick fermentors

Step no.	Growth period (days)	Amount of inoculum	Procedure of step
1	2	25 ml	2 ml of frozen inoculum was added to 25 ml of medium in 100-ml Spinner bottle
2	2	50 ml	25 ml of inoculum from Step 1 was added to 25 ml of medium in 100-ml Spinner bottle
3	1	100 ml	50 ml of inoculum from Step 2 was added to 50 ml of medium in 100-ml Spinner bottle
4	1	200 ml	100 ml of inoculum from Step 3 was added to 100 ml of medium in 500-ml Spinner bottle
5	1	400 ml	200 ml of inoculum from Step 4 was added to 200 ml of medium in 500-ml Spinner bottle
6	1	800 ml	400 ml of inoculum from Step 5 was added to 400 ml of medium in 1-liter Spinner bottle
7	1	2 liters	800 ml of inoculum from Step 6 was added to 1,200 ml of medium in 4-liter Spinner bottle
8	1-2	6 liters	1,000 ml of inoculum from Step 7 was added to 4,000 ml of medium in 7.5-liter New Brunswick fermentor
9	1-2	12 liters	6,000 ml of inoculum from Step 8 was added to 6,000 ml of medium in 14-liter New Brunswick fermentor

(iv) **Polymerase assay.** When JLS-V9 cells in 14-liter suspension units were sampled for presence of DP activity, 500 ml of culture suspension was sampled from each unit and clarified by centrifugation at  $1,000 \times g$  for 20 min. The supernatant fluid containing the virus was then centrifuged at  $105,000 \times g$  for 1 h. The virus pellet was resuspended in Tris-NaCl-ethylene-diaminetetraacetic acid (TNE) buffer (6) to a  $\times 500$  concentration and maintained at  $-70$  C until tested.

Spinner culture samples were prepared for DP activity assay by clarification at  $1,000 \times g$  for 10 min, followed by two subsequent centrifugations of the supernatant fluid at  $10,000 \times g$  for 15 min and  $105,000 \times g$  for 60 min, respectively. The final virus pellet was resuspended in 1 ml of TNE buffer. The virus ( $\times 100$ ) was then assayed for DP activity by use of the following procedure. The standard assay mixture in 150  $\mu$ liters contained 10  $\mu$ mol of Tris-hydrochloride (pH 8.0), 0.1  $\mu$ mol of Mn<sup>2+</sup>, 0.5  $\mu$ mol of Mg<sup>2+</sup>, 0.1  $\mu$ mol of dithiothreitol (DTT), 10  $\mu$ mol of KCl, 10 nmol

each of deoxyadenosine, deoxyguanosine, and deoxycytidine 5'-triphosphate (dATP, dGTP, dCTP), and 1 nmol of tritiated thymidine triphosphate ( $^3\text{H-TTP}$ , 640 counts per min per pmol). The reaction was initiated by the addition of 50  $\mu\text{l}$  (30 to 40  $\mu\text{g}$  of protein) of virus lysate. The virus was lysed with 1% Triton X-100 containing 0.01 M DTT. The mixture was incubated at 37 C for 30 min. The synthesized DNA was precipitated with 10% cold trichloroacetic acid at 0 C. The whole mixture was filtered through membrane filter paper (Millipore Corp., HAWP, 25 mm, 0.45  $\mu\text{m}$  pore size), washed with 5% trichloroacetic acid, dried, and counted in a Beckman scintillation counter (L-355).

**Concentration and purification.** Culture fluids containing RLV were clarified in a Sorvall SS-4 centrifuge with a continuous-flow rotor at  $1,000 \times g$  and concentrated by continuous-flow-with-banding ultracentrifugation in either a model K or model RK zonal ultracentrifuge (Electro-Nucleonics, Inc.). Equal volumes of 20 and 60% (wt/wt) sterile ribonuclease (RNase)-free buffered sucrose were employed in establishing a step-gradient within the rotors prior to controlled acceleration to  $90,000 \times g$ . Peristaltic pumps were employed to pump virus-containing fluids through the rotors at flow rates of 12 and 6 liters/h for the model K and RK ultracentrifuges, respectively. After the continuous-flow operation, rotors were maintained at  $90,000 \times g$  for an additional 30 min to allow the last virus entering the rotor to band isopycnicly. The rotors were then carefully decelerated to rest and were unloaded while the gradient was monitored at 254 nm with a continuous-flow spectrophotometer (Instrumentation Specialities, Co.). The virus zones, after collection, were diluted to 29% sucrose with TNE buffer, pH 7.2, and layered above 30 and 45% sucrose zones in a Beckman TI-15 zonal rotor with B-29 core. The rotor was accelerated to  $95,400 \times g$  in a model L3-50 ultracentrifuge and allowed to operate for 2 h. After deceleration to  $9,540 \times g$ , the gradient was collected (heavy end first) and monitored as above. Sucrose was removed from the virus concentrates by pelleting in preparative ultracentrifuges. Virus pellets were resuspended in TNE buffer at pH 7.2. Virus concentrates were stored at  $-70 \text{ C}$  for subsequent quality control testing.

## RESULTS AND DISCUSSION

**Adaptation of JLS-V9 cells to suspension culture.** The JLS-V9 cells were adapted to growth in suspension culture as follows. A 100-ml amount of Eagle MEM Spinner medium in a 200-ml centrifuge Spinner bottle was inoculated with  $5 \times 10^5$  noninfected monolayer-produced JLS-V9 cells per ml. The cells were observed daily and transferred at 2-day intervals to maintain a cell density of  $2 \times 10^5$  to  $5 \times 10^5$  viable cells per ml. The suspension was trypsinized when necessary to separate large clumps of cells and reconstituted using fresh medium. Cells were propagated for 2 weeks in the MEM Spinner medium, and then the me-

dium was replaced by RPMI 1640. Subsequently a marked increase in growth was noted. After a 4-week period, the cells were well adapted to suspension growth. At this time 1-ml samples of concentrated cell suspension in complete 1640 medium containing 10% dimethyl sulfoxide were frozen by means of control rate freezing (1 C/min) and held at nitrogen vapor temperature.

**Infection of JLS-V9 tissue cells.** A vial of frozen, suspension-adapted JLS-V9 cells was reconstituted and grown in a 100-ml Spinner vessel to a viable cell count of  $7 \times 10^5/\text{ml}$ . The cell concentration of the culture was then adjusted to  $2 \times 10^5$  viable cells/ml. A 25-ml amount of cell suspension and a 3-ml amount of RLV ( $10^7$  particles/ml) produced in monolayer-grown cells were placed in a 100-ml Spinner vessel and incubated at 37 C for 24 h. The cells were pelleted at  $1,000 \times g$  for 20 min, washed with phosphate-buffered saline, and reconstituted with fresh RPMI 1640 medium to 25 ml. The cells were grown to a final volume of 500 ml by four serial transfers, using increasingly larger Spinner flasks. Table 2 shows increased VPC with subsequent transfers postinfection.

After four transfers the infected cells were found to produce virus titering  $10^8$  to  $10^9$  VPC/ml. In subcultures through passage 16, the VPC remained high. These data provided the necessary information for scale-up to a 14-liter New Brunswick unit. In this system, cultures of infected cells were initiated from frozen seed ampoules and grown through the required number of transfers (Table 1) to provide the necessary volume of culture for eventual use in a 14-liter New Brunswick unit.

**Growth of RLV in a 7.5-liter new Brunswick fermentor.** The capacity of JLS-V9 infected cells to propagate RLV in 7.5-liter suspension cultures is demonstrated in Fig. 1. The

TABLE 2. Postinfection virus particle count of Rauscher leukemia virus in suspension culture

Transfer no. postinfection	Cells/ml $\times 10^5$ at sampling time	EM <sup>a</sup> count $\times 10^{10}$ on $\times 100$ concentrates
4	6.0	0.7
8	7.1	7.5
9	6.1	3.7
10	7.6	11.0
11	7.0	14.0
13	7.5	TNTC
15	4.9	3.9
16	—	14.0

<sup>a</sup> EM, Electron microscope.

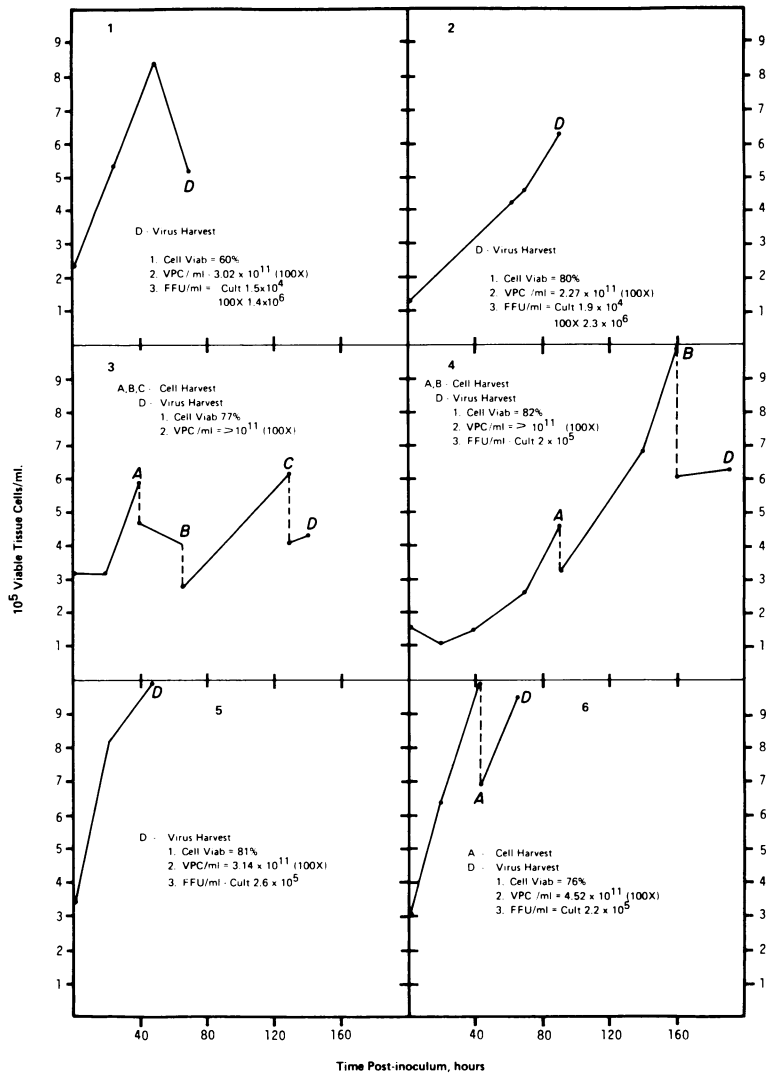


FIG. 1. Growth parameters in 7.5-liter New Brunswick fermentors of Rauscher leukemia virus in infected JLS-V9 suspension cells.

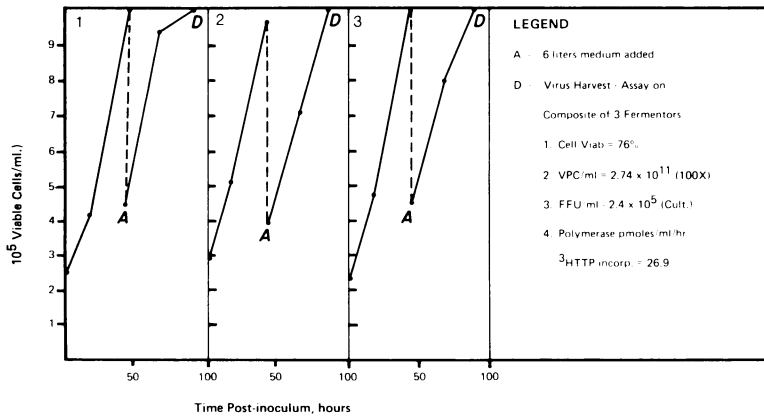


FIG. 2. Growth parameters of Rauscher leukemia virus-infected JLS-V9 suspension cells in 14-liter New Brunswick fermentors.

cultures were initiated with concentrations of cells ranging from  $10^5$  to  $4 \times 10^5$  viable cells per ml. There was an apparent difference in growth rate of the cells in the respective fermentors, due possibly to variations in medium lot and manufacturer. Inasmuch as cells from 7.5-liter fermentors were used both as inocula for the 14-liter fermentors and for production of virus, fermentor runs 3, 4, and 6 demonstrated the repeated capacity to initiate viable cell growth when inoculum was withdrawn and medium was replaced. It was found in 7.5-liter fermentors, sparged with 5%  $\text{CO}_2$  plus 95% air and agitated at 100 rpm, that a pH of 6.7 to 7.0 was maintained and ORP was maintained at  $50 \pm 15$  mV throughout the entire growth period. No alterations of ORP or pH adjustment of the growth medium was necessary during the fermentation.

The harvest from the 7.5-liter fermentors titered greater than  $10^{11}$  VPC/ml using  $\times 100$  concentrates. This yield of virus represents a 3- to 10-fold increase in VPC and a 50-fold increase of in vivo infectivity (FFU/ml) as compared to production lots derived from a roller bottle system in this laboratory during the past 11 months.

**Growth of RLV in 14-liter New Brunswick units.** The cell growth was extremely rapid in the 14-liter units (Fig. 2). Densities of  $10^6$  cells per ml were readily obtained. As in 7.5-liter fermentors, VPC and infectivity in a 14-liter unit were high in the combined composite sample and showed consistently higher yields than those obtained in the roller bottle system. Under the test conditions, which included transfer of spent medium and metabolic by-products with the inoculum, RNA-dependent DNA polymerase (RDDP) activity in produced RLV was low (26.9 pmol per ml per h).

The problems involved in producing fluids of high virus titer in 14-liter New Brunswick units were minimal. These findings permit more efficient large-scale production, in shorter periods of time, than other conventional systems, at reduced cost in terms of growth medium and labor.

**Polymerase activity.** DP activity was found to be associated with early virus synthesis. Using centrifuged actively growing young cells resuspended in fresh medium, DP activity in three replicate Spinner culture experiments peaked during early viral synthesis (Fig. 3). The peak activity occurred when the cell densities reached  $5.0 \times 10^5$  to  $5.5 \times 10^5$  viable cells per ml (approximately 30 h postinoculation).

For characterization of the RDDP activity,

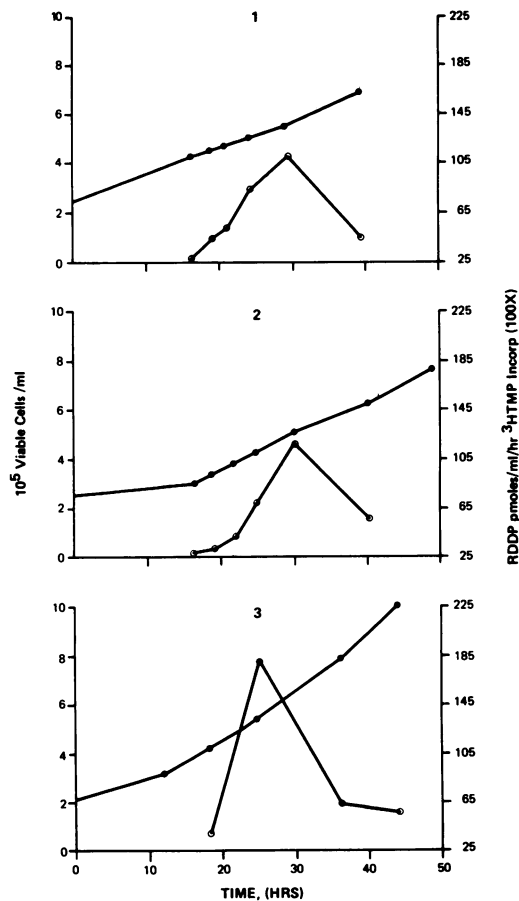


FIG. 3. Cell count and polymerase activity of Rauscher leukemia virus-infected JLS-V9 cells in suspension culture. Symbols: viable cells, ●; RNA-dependent DNA polymerase activity, ○.

DP was assayed under standard conditions with and without lysing the virus. Very little activity was found when the virus was not lysed. The incorporation of  $^3\text{H-TMP}$  with virus lysate was linear up to 30 min and then leveled off (Fig. 4). RLV-DP was also assayed in the absence of some components of the standard enzyme mixture. The results (Table 3) show that  $\text{Mn}^{2+}$  is essential to the reaction. The DTT and KCl enhanced the reaction.  $\text{Mg}^{2+}$  inhibited the reaction, as was earlier reported by Friedlander et al. (2). The presence of an energy generating system, i.e., ATP, did not increase the rate of incorporation of labeled nucleotide into DNA or the amount of DNA synthesized.

The product obtained after 30 min of incubation was further incubated with RNase, deoxyribonuclease (DNase), and NaOH, respec-

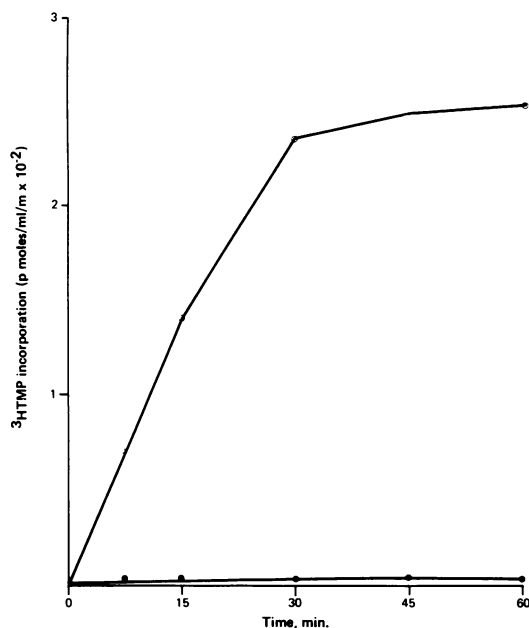


FIG. 4. Incorporation of radioactivity from  $^3\text{H}$ -TTP by Rauscher leukemia virus DNA polymerase. DNA polymerase assays were done as recorded in Materials and Methods at various time intervals as indicated. Symbols:  $^3\text{H}$ -TTP incorporation with virus lysate, O; and without lysing the virus, ●.

TABLE 3. Assay requirements for viral DNA synthesizing system

Assay mixture	Product ( $^3\text{H}$ -TTP incorporation, pmol/ml/h)
CS <sup>a</sup> .....	200
CS - Mn <sup>2+</sup> .....	30
CS - Mg <sup>2+</sup> .....	225
CS - KCl .....	95
CS - DTT .....	84
CS + ATP .....	152

<sup>a</sup> Complete assay mixture (CS) was presented in Method and Materials section.

tively. Table 4 shows that the reaction was not sensitive to NaOH or RNase, but was sensitive to DNase, thereby identifying the labeled product as DNA. The viral enzyme required the three deoxyribonucleotide triphosphates other than labeled TTP in order to synthesize DNA. An 80% decrease in incorporation resulted when the reaction mixture did not contain the three deoxynucleotide triphosphates. In addition,

preincubation of virus lysate with RNase decreased the incorporation of labeled nucleotide by 80%, indicating that the template read by the enzyme was highly sensitive to RNase. The above data indicate that the incorporation of labeled nucleotide into acid-precipitated DNA was mainly due to RDDP.

**Virus concentration and purification.** In three repetitive runs, 30-liter lots of suspension-produced RLV were concentrated and purified by continuous-flow-with-banding and semi-isopycnic ultracentrifugations. The ultraviolet (UV) absorbance profiles (254 nm) of typical runs are presented in Fig. 5A and B. A major UV absorbing peak was obtained from each lot after the initial concentration by continuous-flow-with-banding ultracentrifugation. The densities at which these peaks occurred varied slightly between the three lots, as is commonly encountered in large-scale production. The virus zones were collected, diluted to 29% sucrose, and processed as previously described in a semi-isopycnic ultracentrifugation. This process relies upon the high sedimentation rate of the virus to effect its separation from slower moving particles, even though the virus is still permitted to semi-isopycnically band after the separation. Analysis of the UV absorbance profiles reveals that the heavier virus, under cen-

TABLE 4. Determination of RNA-dependent DNA polymerase activity and product characterization

Reaction conditions	Activity (pmol/ml/h)
Complete system .....	200
Product treated with	
RNase <sup>a</sup> .....	195
DNase <sup>b</sup> .....	32
NaOH <sup>c</sup> .....	186
Viral enzyme preincubated with RNase <sup>c</sup> ...	40
Complete system lacking dATP, dGTP, dCTP <sup>d</sup> .....	42

<sup>a</sup> Thirty-minute product was treated with 10  $\mu\text{g}$  of RNase and further incubated for 45 min. The whole material was then precipitated with 10% trichloroacetic acid at 0 C. The precipitate was filtered and washed with 5% trichloroacetic acid and counted.

<sup>b</sup> As in footnote a, the 30-min product was exposed to 10  $\mu\text{g}$  of DNase/250  $\mu\text{mol}$  of NaOH for 45 min, precipitated with 10% trichloroacetic acid, washed with 5% trichloroacetic acid, and counted.

<sup>c</sup> Viral lysate was incubated with 10  $\mu\text{g}$  of RNase/50  $\mu\text{g}$  of viral protein for 30 min at 37 C. Then 20 to 30  $\mu\text{g}$  of incubated viral protein was used for assay.

<sup>d</sup> The standard mixture for polymerase assay containing no dATP, dGTP, or dCTP was used for this assay.

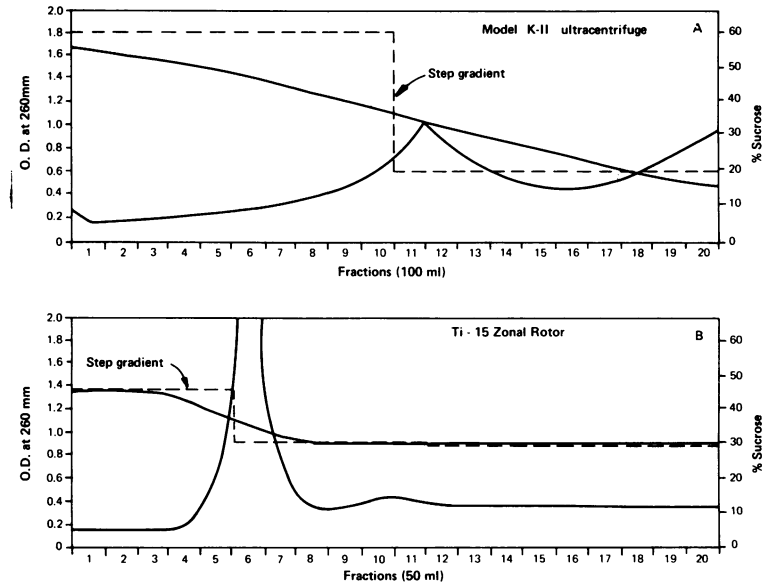


FIG. 5. UV absorbance profiles (260 nm) of suspension culture-produced Rauscher leukemia virus after continuous-flow-with-banding (A) and rate-zonal-with-banding ultracentrifugation (B).

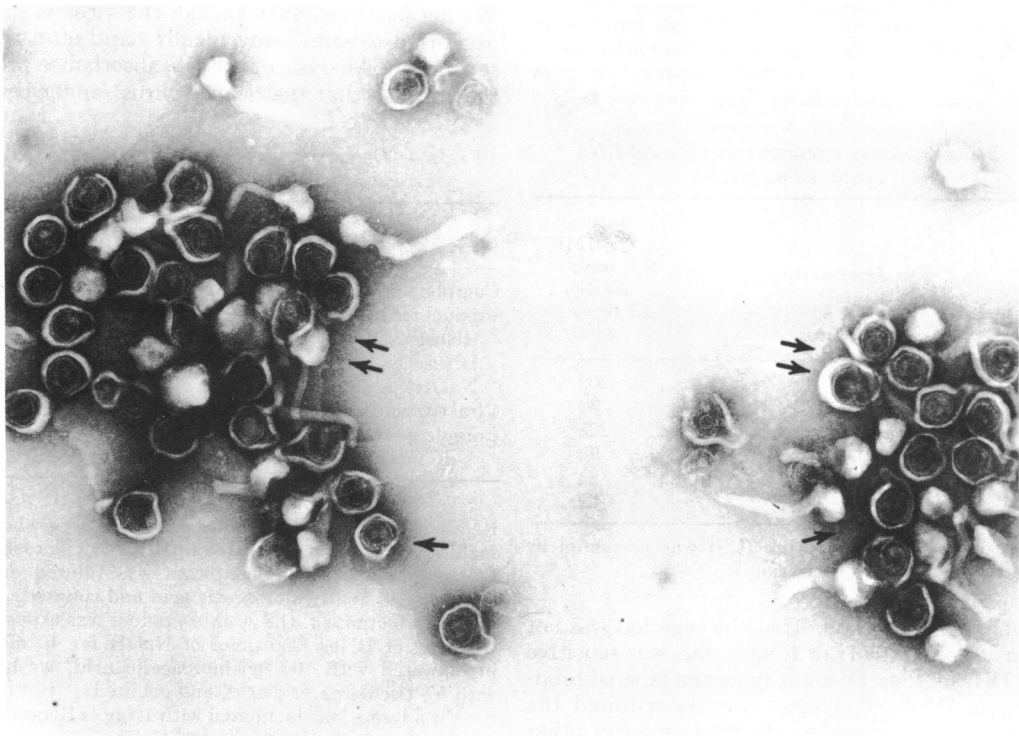


FIG. 6. Micrograph demonstrating the typical morphology of intact Rauscher leukemia virus virions (arrows) produced in JLS-V9 suspension system. Magnification,  $\times 56,000$ .

trifugal force, migrates from the sample zone in the rotor and bands in a very well-defined peak

at a density of 1.14 to 1.16. After collection, the virus was pelleted and resuspended in buffer.

TABLE 5. Quality control testing of concentrated Rauscher leukemia virus produced in suspension cultures

Product lot	Final concn	VPC/ml	In vivo titer (FFU/ml)	Protein <sup>a</sup> (mg/ml)	GS antigen <sup>b</sup> (reciprocal CF titer)	RDDP activity (pmol of <sup>3</sup> H-TMP incorporated/ml/h)
A	×760	1.6 × 10 <sup>11</sup>	1.2 × 10 <sup>6</sup>	0.37	128	20
B	×545	3.3 × 10 <sup>11</sup>	3.2 × 10 <sup>6</sup>	2.18	4,096	0 <sup>c</sup>
C	×290	5.1 × 10 <sup>11</sup>	3.1 × 10 <sup>6</sup>	0.79	256	16.2

<sup>a</sup> Assayed as milligrams per milliliter of protein by the procedure of Layne (5).

<sup>b</sup> Activity as reciprocal complement fixation (CF) titer by procedures outlined in the Army Technical Manual TM8-327-1 (4). GS, Group specific.

<sup>c</sup> The loss of endogenous RDDP activity can be attributed to either template or enzyme inactivation during processing of the material.

Electron photomicrographs show the virus concentrates to be highly concentrated and relatively pure preparations with the integrity of the virions largely intact (Fig. 6).

Quality control testing results on three typical lots are listed in Table 5. The virus concentrates retained the high particle counts and in vivo infectivity associated with the original, unconcentrated material from 14-liter fermentor runs. The above data indicate that the concentration and purification of suspension culture-produced RLV by the methods described above inflicted no significant damage to the virus and necessitated no special treatment in purification over that employed for RLV produced in roller bottle or static culture systems.

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