

## Method for Reproducible Large-Volume Production and Purification of Rauscher Murine Leukemia Virus

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Rauscher murine leukemia virus was produced in roller-bottle cultures of chronically infected JLS-V9 cells. Virus from this culture fluid was concentrated and purified by two semi-isopycnic bandings in sucrose gradients. Virus material obtained from young, nonconfluent cultures (early-harvest virus) yielded products characteristically containing endogenous ribonucleic acid-dependent deoxyribonucleic acid polymerase with high specific activity (400 to 1,000 pmol of [<sup>3</sup>H]thymidine 5'-triphosphate incorporated per milligram of protein per hour). Fluids obtained from older confluent cultures (late-harvest virus) yielded products with endogenous ribonucleic acid-dependent deoxyribonucleic acid polymerase with little or no specific activity (200 pmol or less of [<sup>3</sup>H]thymidine 5'-triphosphate incorporated per milligram of protein per hour), but with higher virus particle counts and greater amounts of protein and gs antigen than the early-harvest products.

As the scope of the National Cancer Institute's Virus Cancer Program expands, the demand for large amounts of purified, oncogenic viruses or suspected oncogenic viruses will increase.

Techniques for the large-scale production and purification of several oncogenic viruses from both monolayer and suspension tissue cultures have been described (5, 10-13). However, one should be aware that no single virus preparation can truly satisfy the various needs of all investigators since the biological and biochemical properties of virus harvests can vary considerably. Harvest intervals of virus-producing cultures must be short (1 to 5 h) to yield preparations with intact 60 to 70S ribonucleic acid (RNA) and high RNA-dependent deoxyribonucleic acid polymerase (RDDP) activity (2, 3), whereas longer intervals (48 to 72 h) are usually required to yield a product with a high viral particle count. With the exception of the report of Toplin and Sottong (13), the reported techniques of virus production appear to lack the flexibility required to easily produce preparations with specifically desired characteristics. In this report, we describe the procedures used in this laboratory to reproducibly produce and to purify large volumes of Rauscher murine leukemia virus (RLV) designed to meet the needs of individual investigators.

### MATERIALS AND METHODS

**Cell and virus stock.** Frozen JLS-V9 cells (14)

chronically infected with RLV were obtained through the Virus Cancer Program, National Cancer Institute, from the John L. Smith Memorial for Cancer Research, Pfizer, Inc., Maywood, N. J. These cells were at the third transfer level postinfection. A portion of these cells was passed three times in our laboratory to yield sufficient cells to establish a 200-ampoule working seed stock which was stored in the vapor phase of liquid nitrogen.

Before using this seed stock, the cells were characterized for cell growth patterns in several media and for virus particle and RDDP yield in a roller-bottle culture system. The seed stock was further examined by conventional methods to verify its freedom from mycoplasma (tested by L. Hayflick) and bacterial and fungal contaminants. Cultured cells from this stock were submitted for mouse antibody production testing (by J. Parker) to verify its freedom from a wide range of viral contaminants (4).

**Reagent quality assurance.** Before use in production, a portion of each reagent lot was subjected to quality assurance testing. All reagents were conventionally tested for aerobic and anaerobic microbial contaminants by using a broad range of media and incubation temperatures. Serum and trypsin lots were tested for mycoplasma and for bacteriophage (7) contaminants.

Medium and serum lots were compared to control lots for their ability to support cellular outgrowth over several transfers with seed stock cells. Before acceptance, lots were required to be at least 75% as efficient at supporting cellular growth as control lots of medium and serum.

**Scale-up and production.** To maintain product integrity, to insure freedom from mycoplasma contamination, and to provide for uniformity, each pro-

duction lot of RLV was initiated from a single ampoule of seed stock. Carefully controlling the culture seeding density at each step, the cells from a single ampoule were scaled-up to production level (Fig. 1) by an initial transfer in static monolayer culture followed by three transfers in roller-bottle monolayer culture. Cells for seeding production-level cultures were divided into three pools to insure that microbial contamination, were it to occur, would not spread throughout the entire lot. The medium used for scale-up was 90% RPMI 1640 (9): 10% heat-inactivated fetal calf serum, supplemented with 200 U of penicillin per ml and 200  $\mu\text{g}$  of streptomycin per ml.

Roller bottles (Bellco Co.) having 1,585  $\text{cm}^2$  of growth surface were used as scale-up culture vessels. These bottles and 10-liter reagent bottles (2,240- $\text{cm}^2$  available growth surface) were used as production-level culture vessels.

Seventy-two hours after the seeding of production cultures, the tissue culture fluids were aseptically harvested within laminar-flow biological safety cabinets by suction supplied by a peristaltic pump. The harvested fluid was pumped through 20 feet (ca. 609.20 cm) of stainless-steel tubing, coiled, and packed in ice for rapid chilling, and then pumped into a sterile 5-gallon (ca. 19-liter) polypropylene carboy also packed in ice. The cultures were refed to original volume with 90% Eagle minimal essential medium, Hanks salts base (HMEM): 10% heat-inactivated fetal calf serum, supplemented with 200 U of penicillin per ml and 200  $\mu\text{g}$  of streptomycin per ml, and were returned to the 37 C incubator. In all subsequent harvests, the production cultures were refed with the HMEM medium.

The RPMI 1640 medium was used throughout scale-up to obtain high cell yields in a short period and to reduce the number of steps to reach production level. During the active virus production step, HMEM was used because it had a better buffering capacity than 1640 medium.

Samples of the harvest material were submitted for microbial contamination testing, virus particle counts (8), and, on certain harvests, RDDP activity by endogenous assay (5). Small amounts of cells and culture fluids were removed from production-level roller bottles and were tested for mycoplasma. A portion of the harvest from every fourth production lot was placed on monolayer cultures of HEp-2 cells and the cultures were examined for cytopathic effects to determine freedom of the harvest from some adventitious viruses.

**Clarification, concentration, and purification.** The harvested tissue culture fluids were immediately clarified of cellular debris by continuous-flow centrifugation in a model J-21 centrifuge (Beckman Inst. Co.) equipped with a JCF-Z zonal rotor. A flow rate of 30 to 35 liters/h at 16,000 rpm was used to subject the harvested fluid to approximately 20,000  $\times g$  for 1.5 min.

Depending upon which viral parameter was being selected for protection, clarified harvests were stored separately at either 4 C (to maintain particle intactness) or -70 C (to protect viral RNA and RDDP activity). Before concentration, frozen harvests were thawed at room temperature, allowing a

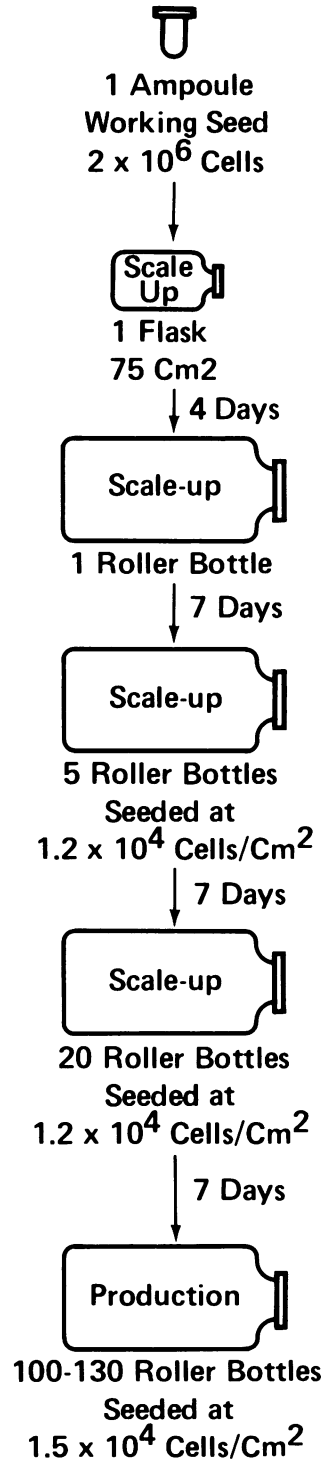


FIG. 1. Scale-up procedure for the production of RLV.

small piece of ice to remain in the 5-gallon (ca. 19-liter) carboy to facilitate refrigeration of the material.

Pools of harvested material were concentrated by continuous-flow-with-banding ultracentrifugation using a model K-II zonal ultracentrifuge (Electro-Nucleonics, Inc.) equipped with either a K-3 or K-6 rotor assembly. With the exception of minor changes, the technique was essentially the same as that described by Toplin and Sottong (13). One liter each of 20 and 60% sterile, ribonuclease-free buffered sucrose (Schwarz/Mann) was used to establish a 20 to 60% sucrose step gradient in the rotor assembly (3.5-liter capacity). Buffers [either TNE buffer: 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.2, 0.1 M NaCl, 0.001 M disodium ethylenediaminetetraacetate; or citrate buffer: 0.05 M sodium citrate, pH 6.9] used in all gradient materials were the same as those in which the final virus concentrates were to be resuspended. After controlled acceleration to 35,000 rpm (90,000  $\times$  *g*), purification lots of 60 to 120 liters of pooled harvest material were forced through the centrifuge by peristaltic pumps at a flow rate of approximately 12 liters/h. After the continuous-flow operation, the rotor was maintained at speed for an additional 30 min to allow the last virus entering the rotor to band semi-isopycally. The rotor was then carefully decelerated to rest and unloaded from the bottom by pumping sterile air into the top of the rotor. The gradient was monitored during unloading with a continuous-flow spectrophotometer (Instrumentation Specialties Co.) at 254 nm. Twenty 100-ml fractions were collected in sterile 4-ounce (ca. 0.118-liter) prescription bottles in wet ice within a laminar-flow biological safety cabinet. The sucrose content of each fraction was determined using an Abbe 3L refractometer (Bausch & Lomb, Inc.) at room temperature. The fractions were then generally allowed to remain overnight embedded in ice within a 4 C refrigerator.

After identification of the virus-containing zone (generally covering densities 1.13 to 1.19 g/cm<sup>3</sup>), the selected fractions from the continuous-flow ultracentrifugation were pooled and were slowly diluted with sterile, cold buffer (4 C) to approximately 26 to 29% sucrose (density <1.14 g/cm<sup>3</sup>). Normally, six 100-ml fractions were selected and were diluted to a total volume of 750 ml. The concentrated virus was then rebanded using a model L3-50 ultracentrifuge (Beckman Inst.) equipped with a Ti-15 zonal rotor with a B-29 core.

A step gradient was established within the Ti-15 zonal rotor by edgeloading the following solutions: 100 ml of buffer, 750 ml of sample, 300 ml of 30% sucrose, and 200 ml of 60% sucrose. After a 2-h ultracentrifugation at 30,000 rpm (84,565  $\times$  *g* maximum), the contents of the rotor were collected in 50-ml fractions by forcing sterile, distilled water into the center of the rotor. This second ultracentrifugation was designed to separate relatively fast-sedimenting particles of density greater than 1.13 g/cm<sup>3</sup> from slower-sedimenting and less-dense particles that remain within the sample zone of the step gradient. At the completion of the run, the gradient

was collected in sterile 2-ounce (ca. 0.059-liter) prescription bottles embedded in ice while being monitored by a continuous-flow spectrophotometer (254 nm).

Relying primarily on ultraviolet absorbance, the virus-containing zone was identified, diluted with sterile, cold buffer, and pelleted by preparative ultracentrifugation using a type 35 rotor (Beckman Inst. Co.). After gentle resuspension in a small volume of the desired buffer, the virus was subjected to two to eight strokes on a tight-fitting, Potter-Elvehjem homogenizer, diluted to the desired volume, dispensed, and stored at -70 C.

Virus pellets were resuspended in either TNE buffer or, in the case of some late-harvest virus products, citrate buffer. Polymerase activity of the final product was not altered by the resuspending buffer. However, stability of gs antigen at -70 C was greater in citrate than in TNE buffer.

**Product characterization.** Frozen (-70 C) samples from each product lot of RLV were assayed for the following. (i) Virus particles were enumerated by the method of Monroe and Brandt (8). (ii) The protein content of the product lot was determined by the method of Lowry et al. (6) using lipid-free bovine serum albumin as the standard. (iii) RDDP activity was assayed as described previously (5). The specific RDDP activity was computed by adjusting the endogenous polymerase activity to reflect activity per milligram of protein. For exogenous activity, dGrC was used as the synthetic template. (iv) The level of MLV gs antigen p30 (1) was determined by complement fixation using rabbit anti-p30 serum. The titer was reported to be the highest twofold dilution of virus (antigen) resulting in complete complement fixation.

## RESULTS

**Effects of culture seeding density, age, and harvest interval on the virus product obtained.** The parameters of cell seeding density, culture age, and interval between harvests of culture fluids were examined to determine their effects on virus particle and RDDP yields. In addition, examination was done to determine how to manipulate these parameters to obtain products with specific, desired characteristics, such as high virus particle counts or high levels of RDDP. The results are shown in Table 1.

Production cultures for lots 6 and 15 were seeded at low cell densities ( $1.5 \times 10^4$  cells/cm<sup>2</sup> of roller-bottle surface). These cultures reached full confluency 120 and 168 h postseeding, respectively. The concentrated and purified material obtained from these cultures 72, 96, and 120 h postseeding, and at a time before their having reached full confluency, contained large amounts of endogenous RDDP (>250 pmol of [<sup>3</sup>H]thymidine 5'-triphosphate incorporated/ml per h), but had moderate virus particle counts ( $6.9 \times 10^{10}$  and  $10^{11}$ /ml, respectively).

TABLE 1. Effect of culture seeding density, age postseeding at time of harvest and interval between harvests on RDDP and virus yield of roller-bottle cultures of RLV-infected JLS-V9 cells

Production lot	Cell seeding density/cm <sup>2</sup> of roller-bottle surface $\times 10^{-4}$	Culture age at harvest (h)	Interval between harvests (h)	Pooled, concentrated (1,000-fold), and purified virus product	
				Endogenous RDDP activity/ml <sup>a</sup>	Virus particle/ml <sup>b</sup>
6	1.9	72-120	24 <sup>c</sup>	280	$1.0 \times 10^{11}$
		240-288	24	22	$2.0 \times 10^{12}$
15	1.5	96-120	24	259	$6.9 \times 10^{10}$
		168-240	24	90	$4.3 \times 10^{11}$
12	3.4	72-120	24 <sup>c</sup>	13	$3.2 \times 10^{10}$
12A	2.5	72-168	48 <sup>c</sup>	32	$1.3 \times 10^{11}$

<sup>a</sup> [<sup>3</sup>H]thymidine 5'-triphosphate incorporated per millimeter per hour.

<sup>b</sup> Electron microscopic virus particle count.

<sup>c</sup> Includes an initial harvest 72 h postseeding.

These same cultures were also harvested, in one case (lot 6), long after reaching confluency (240, 265, and 288 h postseeding) and, in the other (lot 15), shortly after reaching confluency (168 and 240 h postseeding). The concentrated preparations from these harvests contained greater numbers of virus particles ( $2.0 \times 10^{12}$  and  $4.3 \times 10^{11}$ /ml, respectively) than did the previous harvests, but the RDDP activity of these late-harvest products was significantly lower ( $<90$  pmol/ml per h). With the exception of the initial harvests at 72 h postseeding and the final harvest of lot 15 at 240 h, all other harvests were made at 24-h intervals (i.e., complete medium exchange).

When cultures (lot 12) were seeded at a higher cell density ( $3.4 \times 10^4$  cells/cm<sup>2</sup>), full confluency was reached by 72 h postinfection. These cultures were harvested at 72, 96, and 120 h at a time when the cultures were confluent and, except for an initial 72-h harvest, were harvested at 24-h intervals. The product of these harvests contained little polymerase activity (13 pmol/ml per h) and only a low amount of virus ( $3.2 \times 10^{10}$ ).

Production cultures for lot 12A were seeded at a moderate density ( $2.5 \times 10^4$  cells/cm<sup>2</sup>) and reached confluency 168 h postseeding. Harvests were made at 72, 120, and 168 h postseeding, or at 72- and 48-h intervals. The concentrated and purified product from these harvests was low in polymerase activity (32 pmol/ml per h) and contained only a moderate number of virus particles ( $1.3 \times 10^{11}$ /ml).

To clarify further what effects culture age (hours postseeding and percentage of cell confluency) have on virus production in multiple-harvested roller bottle cultures, each harvest of several production lots was monitored for RDDP activity and virus-particle count. The

roller-bottle cultures were also graded for percentage of cell-sheet confluency at each harvest. The results of a typical production lot are shown in Fig. 2. The virus-particle count varied directly with the percentage of cell confluency for the entire 10-day period; i.e., as the culture age and the percentage of cell confluency increased, the virus particle counts of the harvests also increased. However, the RDDP activity increased proportionately with the percentage of cell confluency only until the cultures were 60 to 70% confluent. At this time the recoverable RDDP activity began to fall dramatically even though the virus particle count continued to increase.

**Production.** Based upon these observations, a standard procedure was established for the large-scale production of two basic types of RLV concentrates, i.e., those characterized by high particle count ( $>1 \times 10^{11}$ /ml) and those characterized by high RDDP activity ( $>200$  pmol/ml per h). Production-level cultures were seeded at a density of  $1.5 \times 10^4$  to  $2.0 \times 10^4$  cells/cm<sup>2</sup> of roller-bottle surface. The cultures were harvested and refed at 72, 96, 120, 144, 168, and 240 h postseeding. Until concentration and purification could be effected, the first four harvests (classified as early harvests) were frozen and stored at  $-70$  C. Storage was usually for no more than 1 week. The last two harvests (classified as late harvests) were stored at 4 C and concentrated the day of the last harvest.

Both early- and late-harvest lots were concentrated and purified in the same manner. The gradient profiles from typical zonal ultracentrifugations used to concentrate and to purify both early- and late-harvest lots are shown in Fig. 3 and 4. As the ultraviolet profiles indicate, the second zonal ultracentrifugation separates faster-sedimenting virions from slower-

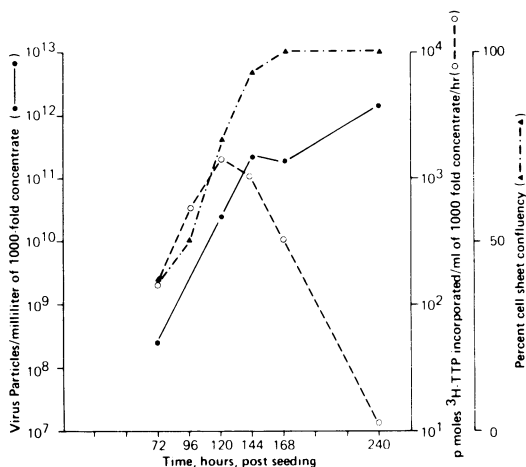


FIG. 2. Correlation between percentage of cell sheet confluency of roller-bottle production cultures harvested and refed at 24-h intervals and the virus particle counts (unpurified samples, concentrated 1,000-fold) and polymerase activities (unpurified samples, concentrated 1,000-fold; [<sup>3</sup>H]thymidine 5'-triphosphate incorporated per milliliter per hour, endogenous assay) of the harvest fluids.

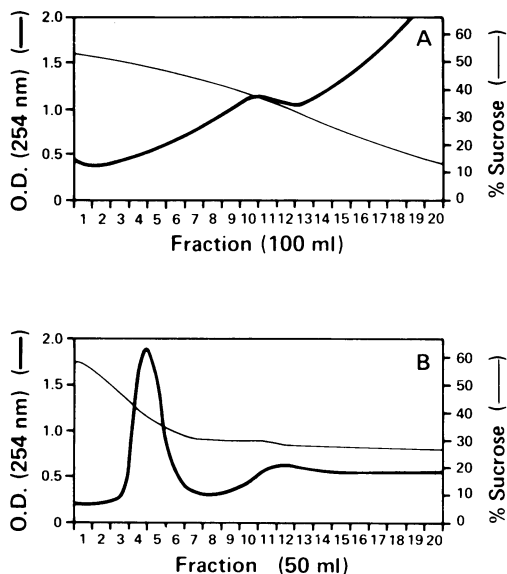


FIG. 3. Typical gradient profiles from (A) continuous-flow recovery (model K-II ultracentrifuge) and (B) rebanding (fractions 8 through 12 rebanded in B) (Beckman Ti-15 rotor, B-29 core) of early-harvest RLV.

sedimenting particles (e.g., ribosomes, etc.) which fail to migrate from the initial sample zone.

The viral particle counts and the specific RDDP activities (RDDP activity per milligram of total protein) of final products derived from

12 consecutive production lots are shown in Fig. 5 and 6. Although most of the product lots had particle counts of 10<sup>11</sup> to 5 × 10<sup>11</sup>/ml, the late-harvest products in most cases had a greater number of virus particles per milliliter than the corresponding early-harvest products.

The specific RDDP activity of the final products (Fig. 6) varied greatly between the early and late products. The activities of the 13 products derived from the early-harvest material exceeded 400 pmol/mg of protein per h in all but two products and exceeded 1,000 pmol/mg of protein per h in seven of the products. With the exception of two product lots (derived from production lots 17 and 26), the late-harvest products contained less than 200 pmol/mg of protein per h of specific RDDP activity.

The exogenous RDDP activity of 14 different final products derived from seven consecutive production lots also varied significantly between early- and late-harvested material. The activities of the products derived from the early-harvest material average about 16,200 pmol/ml per h, whereas the activities of the products from late-harvest virus averaged only 38 pmol/ml per h.

The gs antigen (p30) titers of the late-harvest products ranged from 1:128 to 1:2048 and were usually four times higher than the titers of the corresponding early-harvest products, which ranged from 1:32 to 1:512. The protein content of

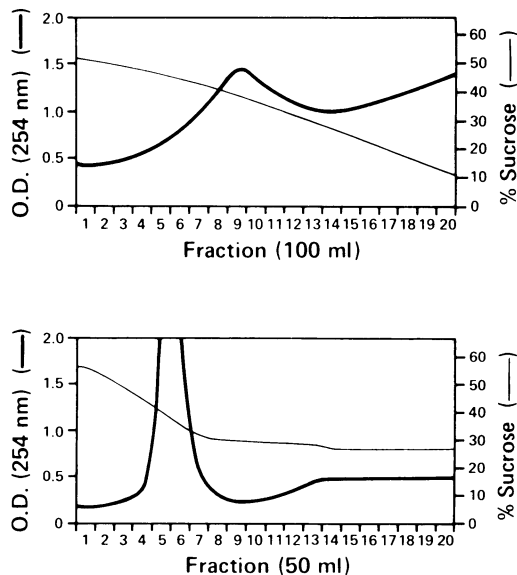


FIG. 4. Typical gradient profiles from (A) continuous-flow recovery (model K-II ultracentrifuge) and (B) rebanding (fractions 7 through 11 banded in B) (Beckman Ti-15 rotor B-29 core) of late-harvest RLV.

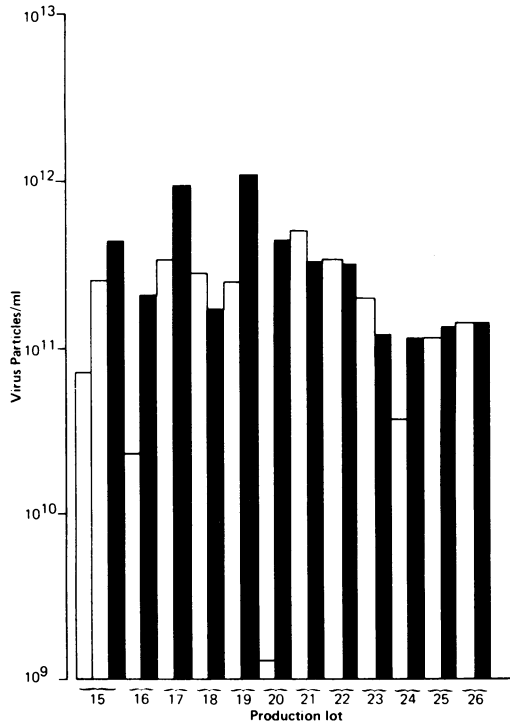


FIG. 5. Virus particle counts of concentrated and purified early- and late-harvested material from 12 consecutive production lots of RLV. Symbols: □, early-harvest virus; ■, late-harvest virus.

the late-harvest products (0.35 to 1.65 mg/ml) were usually 0.3 to 0.5 mg/ml higher than that of the corresponding early-harvest products (0.3 to 1.10 mg/ml).

The recovery of these particles after concentration and purification was equal to or greater than 80% (Table 2). Although a significant amount of RDDP activity was lost during processing, the specific RDDP activities of the products were increased 1,500- to 4,300-fold due to a concomitant greater than 4,300-fold reduction in protein content.

## DISCUSSION

A method for large-volume production of purified RLV products with significantly different biological and biochemical characteristics was described. The differences were expressed as quantitative differences in virus particles present, in endogenous and exogenous polymerase activity, and in total protein and gs antigen content of the final virus products. The differences appear to depend largely on the age of the cultures from which the virus-containing fluids were obtained. Virus harvested from young, actively growing, nonconfluent cultures which,

after an initial medium change at 72-h post-seeding, were harvested and refed with fresh medium every 24 h, yielded preparations which contained moderate levels of virus particles and high levels of specific RDDP activity. This high

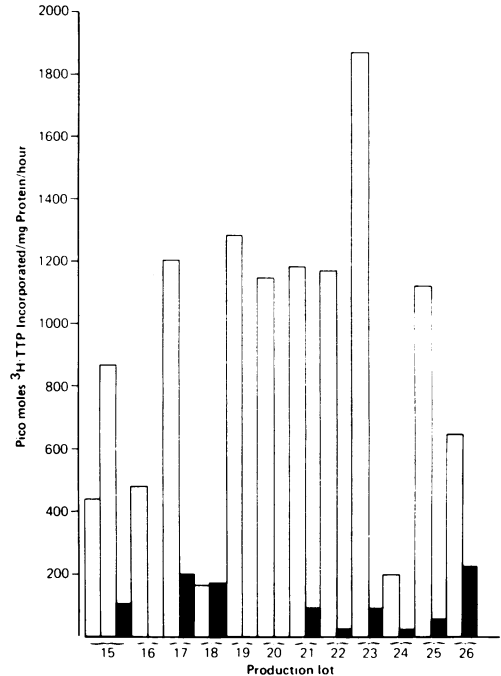


FIG. 6. Endogenous RDDP activity per milligram of protein of concentrated and purified early- and late-harvest material from 12 consecutive production lots of RLV. Symbols: □, early-harvest virus; ■, late-harvest virus.

TABLE 2. Typical recovery of Rauscher murine leukemia virus after concentration and purification by two semi-isopycnic bands of sucrose

Harvest material	Parameter	Harvested tissue culture fluid <sup>a</sup>	Final (1,000-fold) concentrated product <sup>c</sup>	Recovery (%)
Early	Virus particles	$2 \times 10^8$	$2 \times 10^{11}$	~100
	Total protein (mg)	3.8	0.59	0.0016
	Endogenous RDDP <sup>b</sup>	0.77	514	67
	Specific endogenous RDDP	0.20	857	NA <sup>c</sup>
Late	Virus particles	$5 \times 10^8$	$4 \times 10^{11}$	80
	Total protein (mg)	3.8	0.86	0.0023
	Endogenous RDDP <sup>b</sup>	0.27	90	33
	Specific endogenous RDDP	0.07	105	NA

<sup>a</sup> Per milliliter.

<sup>b</sup> Picomoles of [<sup>3</sup>H]thymidine 5'-triphosphate incorporated per milliliter per hour by endogenous assay.

<sup>c</sup> NA, Not applicable.

specific activity reflected both a high endogenous RDDP activity and a low protein content of the purified preparations.

Conversely, material obtained from older confluent cultures yielded preparations with little specific RDDP activity, reflecting both low endogenous RDDP activity and higher protein content. However, these latter preparations did contain relatively greater amounts of virus particles, gs antigen, and protein than did preparations from younger cultures.

The difference in the polymerase activities of the early and late harvests was probably due to an absence of the RDDP enzyme and not to loss of template, possibly through ribonuclease action. This was demonstrated by the near absence of RDDP activity in the exogenous reaction with late-harvest virus even when an artificial template, dGrC, was supplied.

When harvests were examined individually rather than as pools of several harvests, the RDDP activity of each succeeding harvest increased until the cultures nearly reached confluency, a time when cellular activity would be expected to decrease. The RDDP activity was then found in decreasing amounts in each subsequent harvest. The virus particle counts of these successive harvests also increased with culture age, but continued to increase even after the RDDP activity of the individual harvests had started to decrease, indicating possibly less dependence on rapid cellular proliferation for virus release than for enzyme synthesis.

For the parameters discussed, the production technique yielded remarkably uniform products. The specific activity of the polymerase from 7 of the 13 products obtained with early-harvest material was ~1,000 pmol/mg of protein per h, whereas the activity of products from late-harvest material was generally less than 200 pmol/mg of protein per h. The virus particle counts of most of the products fell within a range of  $10^{11}$  to  $5 \times 10^{11}$  particles per ml, regardless of origin. This product uniformity is attributable, in part, to the initiation of each production lot from a single ampoule of working seed, to controlling the culture seeding densities of both scale-up and production-level cultures, and to harvesting production cultures which were always at the same transfer level postinfection.

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