

Purification of Large Amounts of Murine Ribonucleic Acid Tumor Viruses Produced in Roller Bottle Cultures

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Murine ribonucleic acid tumor viruses and C-type virus particles are produced in relatively large quantities in roller bottle cultures. The viruses present in large volumes of culture fluids can be purified by a simple two-step procedure involving polyethylene glycol precipitation and equilibrium centrifugation in sucrose density gradients.

Ribonucleic acid (RNA) tumor viruses contain a 60 to 70S RNA (2) and RNA-dependent deoxyribonucleic acid (DNA) polymerase (1, 10). The demand for purified RNA tumor viruses has greatly increased because of their oncogenicity in animals and because of the interest in the RNA-dependent DNA polymerase. In this investigation, we report on a method to produce and partially purify relatively large amounts of RNA tumor viruses.

MATERIALS AND METHODS

Materials. Fetal calf serum and tylocine were purchased from Grand Island Biologicals, Grand Island, N.Y. Sodium dodecyl sulfate (SDS) was obtained from Matheson Scientific, Houston, Texas; Liquiflour and uridine-5-³H from New England Nuclear Corporation, Boston, Mass.; Triton X-100 from Beckman Instruments, Fullerton, Calif.; polyethylene glycol (PEG) 20 M from Union Carbide, Houston, Texas; membrane triacetate filters, type GA-6, 25-mm diameter from Gelman Instrument Company, Ann Arbor, Mich.; and 2-quart mold-blown glass disposable bottles and 4- or 9-tier Rollacell tissue culture units from New Brunswick Scientific, New Brunswick, N.J.

Cells and virus. The original stocks of JLS-V5, JLS-V6, JLS-V9, and JLS-V10 cells were supplied to us by R. L. Tyndall, Oak Ridge National Laboratory, Oak Ridge, Tenn. Rat embryo fibroblasts which produce murine sarcoma virus (SD-MSV) were obtained from James East, Department of Virology, The University of Texas at Houston M. D. Anderson Hospital and Tumor Institute, Houston, Texas. The JLS-V5 cells were the source of Rauscher murine leukemia virus (RLV).

Preparation of stock solutions and culture medium. The concentrations of salts and glucose in stock solutions are as described (5) using an Earle's

base. The Eagle amino acid formula (5) has been modified to contain twice the usual concentration of amino acids except for histidine (1.68 g/40 liters), glycine (1.20 g/40 liters), serine (1.68 g/40 liters), and arginine (3.36 g/40 liters). The salts-glucose-amino acid mixture was prepared in 40-liter quantities containing 2.0 ml of 10% (w/v) phenol red, pH 7.5, filter sterilized, and stored in 1,600-ml quantities at room temperature. Stock solutions for 100× Eagle glutamine (Nutritional Biochemicals, Cleveland, Ohio) and 100× Eagle vitamins (Difco Laboratories, Detroit, Mich.) were filter sterilized and stored at -20 C. Other stocks include a filter-sterilized 7.5% NaHCO₃ solution and an autoclave sterilized 10× (3.3%) Tryptose phosphate broth (Difco Laboratories, Detroit, Mich.). These stocks were stored at 4 C and room temperature, respectively. A 25% neomycin sulfate (Upjohn Drug Company, Kalamazoo, Mich.) solution was stored at 4 C.

The complete culture medium was prepared immediately prior to use as described in Table 1.

C-particle virus purification. Culture fluids from the fifth or sixth day after subculturing were used as a source of C-particles. The medium was clarified by a low-speed centrifugation (2,500 × g for 10 min). The supernatant fluid was made 6% (w/v) in PEG 20 M and stored at 4 C overnight. The precipitate was collected by centrifugation at 10,000 × g for 30 min, and resuspended by homogenization in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, 0.1 M NaCl, and 0.001 M ethylenediaminetetraacetic acid (TNE) (1/100 of the original culture fluid volume). About 8 ml of this suspension was warmed at 25 C for 5 min and was layered on 28 ml of a 15 to 60% (w/v) linear sucrose gradient buffered in TNE. After centrifugation for 16 hr at 13,000 rev/min in a Beckman SW-27 rotor, the virus was concentrated in a sharp band in the 1.15 to 1.16 g/cm³ region of the gradient. The opalescent virus band was removed with a Pasteur pipette, diluted three to fivefold with TNE, and collected by

TABLE 1. Recipe for complete culture medium (2 liters)

Additions	Volume (ml)
Salts, glucose, and amino acid mixture . . .	1,600
Fetal calf serum	200
10 × Tryptose phosphate broth	200
7.5% (w/v) Sodium bicarbonate	60
100 × Glutamine	40
100 × Vitamins	20
Tylocine, 6,000 µg/ml	40
Neomycin sulfate, 200 mg/ml	1

centrifugation at $78,000 \times g$ for 90 min.

To compare the yield of RLV from PEG precipitation of culture fluids to the yield by conventional high-speed centrifugation, 400 ml of 24-hr culture fluid from JLS-V5 cells labeled with ^3H -uridine were divided into two equal portions. Half of the clarified culture fluid was centrifuged at $10,000 \times g$ for 30 min and the resulting supernatant fluid was centrifuged at $78,000 \times g$ for 90 min. Each pellet was dissolved in TNE and applied to a 15 to 60% linear sucrose gradient, and the virus was isolated by equilibrium centrifugation. The remaining portion of the clarified culture fluid was precipitated with 6% (w/v) PEG and processed in the same manner.

Recent studies have shown that dextran sulfate 500 (Pharmacia Fine Chemicals, Uppsala, Sweden), when added during the purification and storage of the virus, stimulates the reverse transcriptase activity about twofold. Dextran sulfate (50 µg/ml) was added to the culture medium prior to the clarification and to all solutions in the subsequent stages of purification. The yield of virus was reproducibly higher (10%) in the presence of dextran sulfate. The virus for reverse transcriptase studies was stored in 0.01 M Tris, pH 8.1, 0.01 M dithiothreitol, and 50 µg of dextran sulfate/ml. The reverse transcriptase activity was stable for several months when stored under these conditions at -60°C . All reverse transcriptase studies were done with virus purified in the presence of dextran sulfate.

^3H -uridine-labeled virions were purified by the same procedure from cells exposed to ^3H -uridine in the presence of µM thymidine. Approximately 200 µCi of uridine-5- ^3H (specific activity 20 Ci/mmmole) was added per roller bottle, with each bottle containing 100 ml of growth medium. Every 24 hr, the culture fluid was harvested and fresh culture medium and isotope were added.

Virus RNA extraction. ^3H -RNA was extracted from the virus with SDS-phenol. The virus was suspended in 2 ml of TNE containing 1% SDS. After 5 min at 25 C, one-half volume of phenol was added and the mixture was shaken for 5 min at 25 C. The mixture was centrifuged at $10,000 \times g$ for 2 min, and the aqueous layer was reextracted with phenol. The RNA was precipitated from the aqueous phase with 2 volumes of alcohol after addition of 1/10 volume of 2 M potassium acetate, pH 5.3. The precipitate was washed several times by suspension in 67% alcohol containing 0.07 M potassium acetate, pH 5.3. The

RNA was dissolved in 0.01 M Tris, pH 7.5, and kept at -60°C until used. Velocity sedimentation analysis of the RNA was performed by centrifugation in a linear 5 to 25% sucrose gradient buffered in TNE for 16 hr at 15,000 rev/min in the Beckman SW-27 rotor. Heat-dissociated RNA was prepared as described previously (2).

Reverse transcriptase assay. A modification of the procedure described by Scolnick et al. (9) was used. The assay contained the following components in 0.1 ml final volume: 0.2 µmole of MnCl_2 , 6 µmoles of KCl; 3.9 µmoles of Tris-hydrochloride, pH 8.1, at 37 C; 0.05 µmole each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 10 µCi of ^3H -thymidine triphosphate (TTP), specific activity 17.8 Ci/mmmole; and 2- to 100-µg quantities of intact virus protein. The intact virus serves as both an enzyme and a template source. No exogenous templates were added. The reaction mixture contained an optimal level of Triton X-100 (0.02%) and was incubated for 60 to 120 min at 37 C and chilled in ice. About 0.1 ml of 0.1 M sodium pyrophosphate, 100 µmoles of TTP, and 2 ml of cold 5% trichloroacetic acid were added. After 20 min at 0 C, the samples were collected on Gelman triacetate filters, and the filters were washed seven times with 5% trichloroacetic acid. The edges of the filters not accessible to trichloroacetic acid washing were trimmed to reduce the background radioactivity.

Liquid scintillation counting. All gradient fractions were processed through a trichloroacetic acid precipitation procedure. Usually 100 µg of yeast RNA was added as carrier, and an equal volume of cold 10% trichloroacetic acid was added. After 20 min at 0 to 4 C, the precipitates were collected on Gelman triacetate filters and the filters were blotted dry and placed in counting vials. One milliliter of 0.4 M NH_4OH and 10 ml of counting fluid were added. The counting fluid contained 1,000 ml of Triton X-100, 1,880 ml of toluene, and 126 ml of Liquiflour. The samples were counted in a Packard 3375 scintillation counter at 28% efficiency for tritium.

RESULTS

Subculturing of cells. This procedure is outlined for JLS-V5, but applies equally well to the other cell lines such as JLS-V6, JLS-V9, or JLS-V10, and to SD-MSV-infected rat embryo fibroblasts. One fully confluent roller culture (7 days old) is drained well and 10 ml of freshly prepared complete growth medium is added. The cells are removed from the glass surface with a sterile rubber policeman. The cell suspension is pipetted repeatedly against the walls of the bottle to disperse clumps of cells. An appropriate volume of dispersed cells is added to the 2-liter volume of complete growth medium. For example, 5 ml of the original 10-ml cell suspension is added to 2 liters of medium and distributed into 20 roller bottles (100 ml each). This represents a

1:40 split. Each bottle is gassed 15 to 20 sec with 10% CO₂ in air. The gassing procedure is only necessary at the initial subculturing stage. The bottles are sealed with rubber stoppers or screw caps and placed on the roller mill. In our experience, the cell line grows very well at 3 to 6 bottle revolutions per hr at 37 C. Faster speeds usually yield smaller numbers of cells per bottle. Culture fluid changes (100 ml) are done on the second, fifth, and sixth days of growth. A workable schedule to use is as follows: the cultures are seeded on Wednesday, and the medium is changed on Friday and the following Monday. Cells are ready to use on Tuesday (day 6); if not, another medium change is required. Subculturing is then done on Wednesday.

A variety of cells have been grown under these conditions using this procedure. The growth conditions produce 1.0 to 3.0 ml of packed cells per 2-quart roller bottle, depending on the cell line. Usually, cells from one confluent roller culture can be seeded into 20 or 40 roller cultures. JLS-V6 cells yield about 1.5 ml of packed cells per roller bottle every 7 days; MSV-infected rat embryo cells yield about 1.5 ml whereas JLS-V5 cells produce 3.0 ml per bottle per week. Table 2 shows the increase of cell volume during a week's growth using JLS-V5 cells. Baby hamster kidney cells (BHK-21, clone 13) yield about 1.0 ml of packed cells per roller bottle every 4 to 5 days at a 1:20 to 1:30 split. The BHK cells are removed from the glass with trypsin instead of by mechanical scraping.

C-particle virus purification. PEG 6 M or 20 M has been shown to precipitate virus particles from solutions (3, 4, 6, 11). We have utilized this technique for the isolation of C-type virus particles from JLS-V6 culture fluids (7), for Rauscher leukemia virus, and for the SD-MSV. Approximately 40 to 60 μ g of RLV-RNA can be obtained per liter of JLS-V5 culture fluid per 24 hr.

The comparison of the RLV yields by PEG 20 M precipitation of culture fluid to the yields by conventional high-speed centrifugation is

TABLE 2. Growth of JLS-V5 cells in roller bottle culture

Days following subculture	Confluency (%)	Packed cell volume (ml)
0		<0.1
2	5 to 10	0.2
5	45	1.1
6	75	2.0
7	100	2.8

presented in Fig. 1A-D. The results show very little virus pellets at 10,000 $\times g$ in the absence of PEG, but a large peak of virus is seen in the 78,000 $\times g$ pellet. However, in the presence of PEG, a quantitative yield of virus is obtained in the 10,000 $\times g$ pellet (Fig. 1C). Very little virus was detected in the supernatant fluid after PEG precipitation (Fig. 1D). The PEG concentrate shows 260 nm absorbing material at the top of the gradient and a homogeneous peak of virus at 1.15 to 1.16 g/cm³ sucrose. Very little trichloroacetic acid-precipitable radioactivity is present at the top of the tube and a radioactivity peak coincides with the 260 nm absorbance peak of virus at 1.15 to 1.16 g/cm³ density (Fig. 1C). When the virus band was pooled, diluted, and refractionated on the sucrose gradient, 90% of the virus banded in the same position. Similar results were ob-

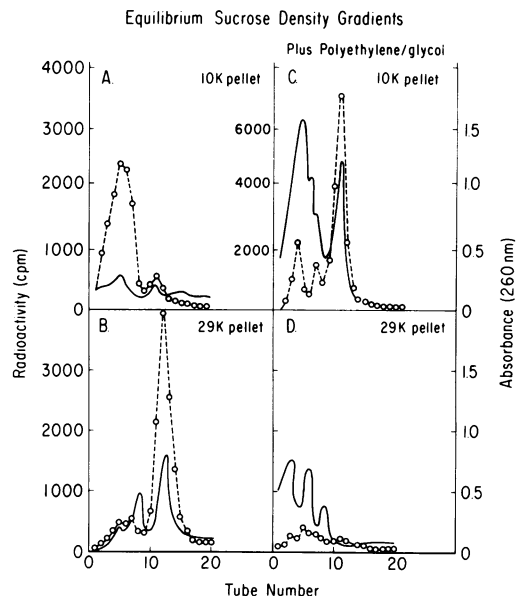


FIG. 1. Comparison of the yield of Rauscher murine leukemia virus as isolated from culture fluids by high-speed centrifugation or polyethylene glycol precipitation. Four JLS-V5 roller bottle cultures were labeled with uridine-5-³H for 24 hr. The culture fluids were processed as described in the text. About 2-ml samples were applied to 15 to 60% linear sucrose gradients (15 ml) buffered in 0.01 M Tris, pH 7.5, 0.1 M NaCl, and 0.001 M EDTA. The tubes were centrifuged in the SW-27 rotor at 13,000 rev/min for 16 hr at 4 C. The direction of sedimentation is from left to right. The 1.16 g/cm³ region of the gradient is approximately tube numbers 12 and 13. The solid lines represent absorbance at 260 nm and the open circled, broken lines represent incorporated ³H-uridine monophosphate radioactivity in counts/min.

tained with the C-particles produced by JLS-V6 cells (7) and with SD-MSV.

The purified RLV and the C-particles produced by JLS-V6 cells (7) were examined by electron microscopy (Fig. 2). The electron micrographs show C-type Rauscher virus particles (Fig. 2A), and similar particles were observed in the preparation purified from culture fluids of JLS-V6 cells (Fig. 2B). Tail particles were observed in both preparations stained with uranyl acetate. Staining in phosphotungstic acid gave fewer tailed particles and more rounded structures.

When 24-hr culture fluids were processed from noninfected BHK-21 cells, a homogeneous optical density peak was seen in the 1.16 g/cm³ region of sucrose gradients. This peak

was about one-fourth the size of the peak from RLV-infected JLS-V5 culture fluids. No detectable labeled RNA was seen in 1.16 g/cm³ peak when the BHK-21 cultures were exposed to ³H-uridine for 24 hr. In addition, no reverse transcriptase activity was present in the 1.16 g/cm³ peak from BHK-21 culture fluids (Fig. 3 and 4), and C-type virus particles were not observed in electron micrographs. The electron micrographs suggest that microsome-like components are present in this fraction from BHK-21 cells. Thus, the 1.16 g/cm³ region of the gradient from culture fluids of C-particle-producing cells probably contains other membrane components in addition to C-type virus particles. These membrane components probably are minor components in high-producer

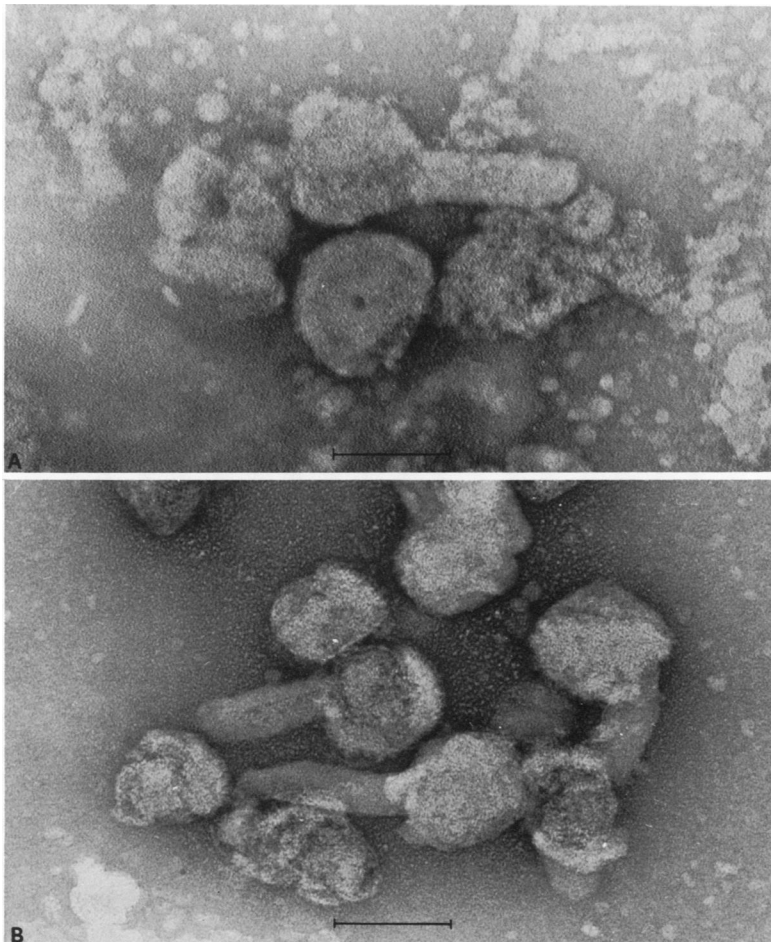


FIG. 2. Electron micrographs of purified Rauscher murine leukemia virus (RLV) from JLS-V5 culture fluids and of the C-particles from JLS-V6 culture fluids. The purified virus particles were applied to a Formvar-coated grid and stained with uranyl acetate. Panel A shows RLV from JLS-V5 culture fluids, and panel B shows the C-particles produced by the JLS-V6 cultures. The scale bar equals 0.1 μ m.

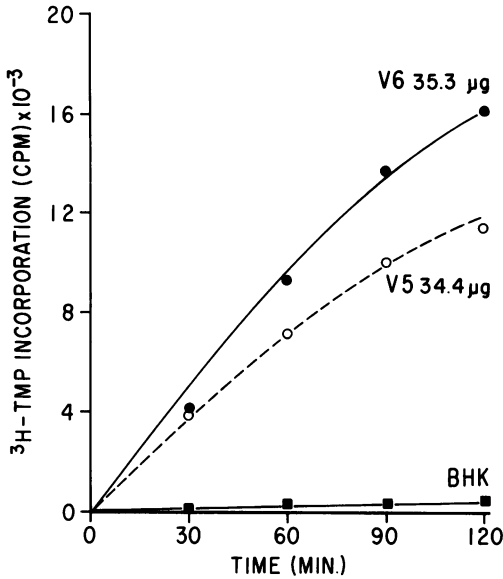


FIG. 3. Kinetics of reverse transcriptase activity of the JLS-V6 and JLS-V5 (Rauscher murine leukemia virus) particles. Purified virions from culture fluids of JLS-V6 and JLS-V5 cells were incubated in the standard assay system with ³H-thymidine triphosphate and the three other deoxyribonucleoside triphosphates at 37 C for the times shown. A fraction purified in the same manner from BHK-21 culture fluids was used as a control.

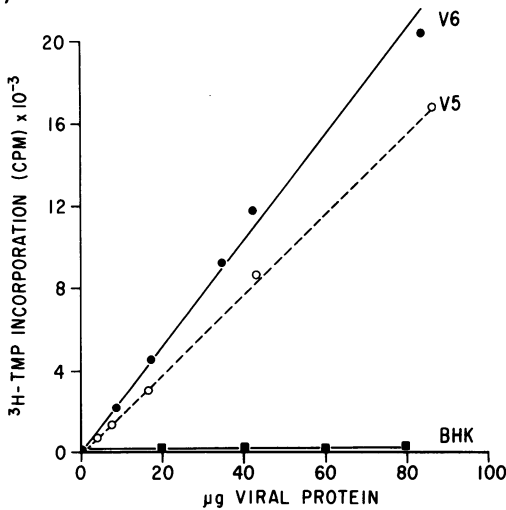


FIG. 4. Concentration dependence of virion protein and reverse transcriptase activity. Various amounts of purified virions from culture fluids of JLS-V6 and JLS-V5 cells (Rauscher murine leukemia virus) were incubated for 60 min at 37 C in standard assay system with ³H-thymidine triphosphate and the three other deoxyribonucleoside triphosphates. A fraction purified from BHK-21 culture fluids was used as a control.

cells such as JLS-V5 cells.

Reverse transcriptase. The purified RLV prepared in this manner contained adequate levels of reverse transcriptase. The synthesis of DNA was dependent on Triton X-100, it was abolished by pancreatic ribonuclease, and it required the three other deoxyribonucleoside triphosphates (Table 3). The product was shown to be DNA by its sensitivity to deoxyribonuclease and 0.5 N HCl, and by its resistance to alkali and pancreatic ribonuclease (Table 3). The time course of synthesis was nearly linear for 2 hr (Fig. 3) and there was a linear dependence on protein concentration (Fig. 4). Similar results were obtained with the C-type virus particle produced by JLS-V6 cells (Fig. 3 and 4). No reverse transcriptase ac-

TABLE 3. Requirements for Rauscher virus ribonucleic acid-dependent deoxyribonucleic acid polymerase and characterization of the product^a

Expt no. ^b	Treatment	[³ H]TMP incorporation (counts/min)
1	Untreated	7,200
	-dCTP	389
	-dGTP	605
	-dATP	295
	-Triton X	335
	10 µg of ribonuclease in reaction mix	540
2	Nucleic acid product	10,800
	+50 µg of ribonuclease	9,720
	+50 µg of deoxyribonuclease	1,415
3	Nucleic acid product	7,200
	+NaOH hydrolysis	6,840
	+HCl hydrolysis	3,096

^a Abbreviations: dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; TMP, thymidine monophosphate.

^b In experiment 1, the standard polymerase assay was performed using 35 µg of viral protein in a 60-min assay. One picomole of [³H]TMP incorporation is approximately 10,000 counts/min. In experiment 2, 70 µg of viral protein was incubated for 60 min at 37 C in a 2× standard reaction mixture. The nucleic product was extracted with SDS-phenol and dissolved in 1 ml of 0.01 M MgCl₂, pH 7.0. The nucleic acid was incubated with 50 µg of appropriate nuclease in a final volume of 0.3 ml for 1.5 hr at 37 C. In experiment 3, standard reaction mixtures were incubated for 60 min at 37 C with 35 µg of viral protein. A sample of the nucleic acid product was boiled 5 min in 0.5 N NaOH or 0.5 N HCl.

tivity was detected in culture fluids from non-infected BHK-21 cells.

RLV-RNA. The ^3H -RNA from Rauscher leukemia virus labeled for 24-hr was extracted with SDS-phenol and analyzed by velocity sedimentation on a sucrose gradient (Fig. 5). The results show a peak of 65S RNA in a 5 to 25% gradient buffered in TNE. About 75% of the RNA sedimented at 65S and the remainder was seen at the top of the tube. When 60 to 70S RNA was heat denatured (2), it had a heterogeneous profile with *S*-rate limits of 4 to 40S. The ^3H -RNA from purified JLS-V6 virus gave similar results (7), but the SD-MSV gave a heterogeneous 60 to 70S RNA peak and the heat-dissociated RNA was more homogeneous.

The ^3H -labeled viral RNA was 95% sensitive to pancreatic ribonuclease in 0.3 M NaCl (10 $\mu\text{g}/\text{ml}$ for 30 min at 37 C), and to 0.5 N NaOH for 5 min at 95 C as measured by trichloroacetic acid precipitation.

When virus was isolated from cultures labeled for 8 and 12 hr, respectively, the heat-dissociated RNA contained a more homogeneous 33 to 38S RNA peak (C. S. Wang and R.

B. Arlinghaus, unpublished observations). Thus, in agreement with the results of Watson (12), the Rauscher leukemia virus 60 to 70S RNA is "nicked" or has single-strand breaks in double-stranded regions during the 24-hr period of labeling.

DISCUSSION

We have described procedures to produce relatively large amounts of C-type virus particles in roller bottle cultures. The use of PEG 20 M precipitation allows concentration of the virus particles from large volumes of culture fluid efficiently and at low costs. When large volumes of fluid are involved, the alternative is to use expensive, continuous-flow ultracentrifuges to concentrate and partially purify C-type viruses. Ammonium sulfate precipitation has been used to concentrate avian RNA tumor viruses (8), but this procedure was found to be detrimental to murine RNA tumor viruses. The use of high CsCl concentrations appears to inactivate the murine RNA tumor viruses (8).

Polyethylene glycol 6 M quantitatively precipitates a number of plant viruses (4) and has been used to precipitate murine leukemia virus (3) and other myxoviruses (6). Foot-and-mouth disease virus (11) can be quantitatively precipitated with PEG 20 M, and we have shown that mengovirus behaves similarly (W. T. Loesch and R. B. Arlinghaus, unpublished results). The results reported here show that murine C-type virus particles are quantitatively precipitated by PEG 20 M (Fig. 1). The precipitated viruses band at 1.15 to 1.16 g/cm^3 , contain 60 to 70S RNA, and have reverse transcriptase activity. The reverse transcriptase activity in the 1.16 g/cm^3 region of the sucrose gradient is indicative of the presence of C-type virus particles. The reverse transcriptase activity in virus purified by PEG 20 M precipitation has the usual requirements and the DNA product has the usual properties (1, 10).

The 60 to 70S RNA of these purified viruses is heat sensitive, producing 35S RNA and lower *S*-rate components (Fig. 5). The C-particles purified from 24-hr culture fluid contains RNA which has been partially "nicked" by nucleases. The 60 to 70S RNA appears relatively normal, but the heat-dissociated RNA is quite heterogeneous. When virus was purified from culture fluids labeled for either 8 or 12 hr, the 60 to 70S RNA appeared to be identical to the 24-hr virus RNA, but the heat-dissociated RNA contained a more homogeneous 33 to 38S RNA. Similar results have been obtained by

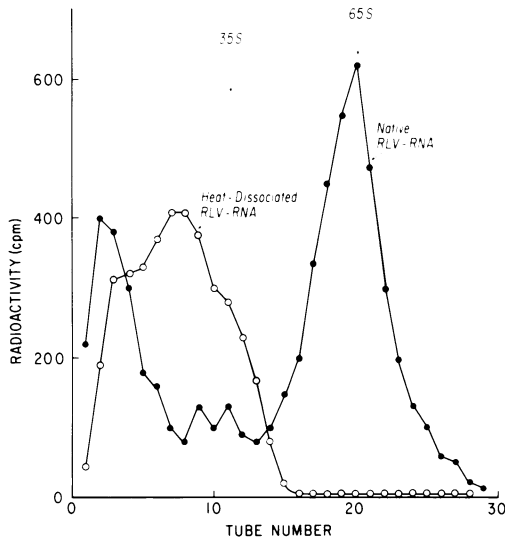


FIG. 5. Rauscher leukemia virus RNA before and after heat treatment. The ^3H -RNA was extracted from purified RLV with sodium dodecyl sulfate-phenol. One sample was applied to a linear 5 to 25% sucrose gradient buffered in 0.01 M Tris, pH 7.5, 0.1 M NaCl, and 0.001 M EDTA. A second sample was heated for 2.5 min at 80 C as described (2) and applied to another sucrose gradient. Both samples were centrifuged at 15,000 rev/min for 16 hr at 4 C in the SW-27 rotor. The direction of sedimentation is from left to right.

Watson (12) with the Rauscher leukemia virus.

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LITERATURE CITED

- Baltimore, D. 1970. Viral RNA-dependent DNA polymerase. *Nature (London)* **226**:1209-1211.
- Blair, C. D., and P. H. Duesberg. 1968. Structure of Rauscher mouse leukemia virus RNA. *Nature (London)* **220**:396-398.
- Chermann, J. C., M. Raynaud, C. Jasmin, and G. Mathe. 1970. Powerful new inhibitor of murine leukaemia and sarcoma viruses. *Nature (London)* **227**:173-174.
- Clark, M. F., and R. M. Lister. 1971. The application of polyethylene glycol solubility-concentration gradients in plant virus research. *Virology* **43**:338-351.
- Eagle, H. 1969. Amino acid metabolism in mammalian cell cultures. *Science* **130**:432-434.
- Kanarek, A. D., and G. W. Tribe. 1967. Concentration of certain myxoviruses with polyethylene glycol. *Nature (London)* **214**:927-928.
- Naso, R. B., J. G. Vidrine, C. S. Wang, and R. B. Arlinghaus. 1972. C-type virus particles produced by normal mouse spleen-thymus cultures. *Virology* **47**:521-524.
- Robinson, W. S., and P. H. Duesberg. 1968. The chemistry of the RNA tumor viruses, p. 306. *In* H. Fraenkel-Conrat (ed.), *Molecular basis of virology*. Van Nostrand Reinhold Co., New York.
- Scolnick, E., E. Rands, S. A. Aaronson, and G. F. Todaro. 1970. RNA-dependent DNA polymerase activity in five RNA viruses: divalent cation requirements. *Proc. Nat. Acad. Sci. U.S.A.* **67**:1789-1796.
- Temin, H. M., and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature (London)* **226**:1211-1213.
- Wagner, G. G., J. L. Card, and K. M. Cowan. 1970. Immunochemical studies of foot-and-mouth disease. VII. Characterization of foot-and-mouth disease virus concentrated by polyethylene glycol precipitation. *Arch. Gesamte Virusforsch.* **30**:343-352.
- Watson, J. D. 1971. The structure and assembly of murine leukemia virus: intracellular viral RNA. *Virology* **45**:586-597.