Production and Purification of Large Amounts of Rous Sarcoma Virus

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Received for publication 15 December 1972

Procedures are described for production and purification of large amounts of Rous sarcoma virus. The virus was produced by Rous sarcoma virus-transformed chicken embryo fibroblasts in roller culture which produced up to 6 mg of virus per day per liter of supernatant fluid. Various methods of concentrating virus were evaluated; pelleting yielded the best results in terms of recovery of infectious virus. Purification was achieved by means of successive velocity and equilibrium density centrifugation by using sucrose solutions made in low-salt buffer. A rapid method for the optical density measurement of virus concentration was also developed.

Research in the biochemistry of ribonucleic acid (RNA) tumor viruses has been restricted to a few strains which are available in large quantities. The most commonly used avian virus is avian myeloblastosis virus (AMV) which, although present in high concentrations in the plasma of leukemic birds, has several disadvantages. Among these are the fact that (i) it is biologically complex, since several viruses are known to exist in standard AMV (20, 23), (ii) as recovered from the plasma, it has a more complex polypeptide composition than virus grown in tissue culture (3), and (iii) it is difficult to assay in vitro in terms of its biological activity. Transformation assays for it are relatively inefficient (2, 19), so that indirect techniques such as interference tests (22) and fluorescent-focus assays (30) must be used for its nontransforming component.

Nondefective Rous sarcoma virus (RSV) strains, on the other hand, have advantages which should aid biochemical characterization. Several strains are available which are nondefective and which have been repeatedly cloned with no evidence for the necessity of an associated leukemia virus for replication. They grow to relatively high titers in tissue culture and retain the ability to transform cells. In addition, the assay for infectious RSV is the simple focus assay (29).

The Prague strain of RSV (Pr-RSV) has several features which make it attractive for large-scale production. In particular, its subgroup C strain will transform a wide variety of commonly available chicken embryo fibroblasts. Further, it is nononcogenic in mammals (9), which minimizes the dangers inherent in working with high concentrations of virus.

This investigation was directed to the following points: (i) the production of Pr-RSV in large amounts, (ii) the evaluation of large-scale methods of virus concentration and purification, and (iii) the rapid and simple measurement of purified virus concentration.

MATERIALS AND METHODS

Virus strains. Pr-RSV, RSV(RAV-1), RSV(RAV-2), and Schmidt-Ruppin RSV subgroup B (SR-RSV-B) were obtained from P. K. Vogt. Strain MC29 was obtained from J. W. Beard, from which a subgroup B virus was isolated and designated MC29-B (unpublished data). AMV, BAI strain A, was obtained from J. W. Beard. L-cell virions were purified from supernatant fluids of mouse L fibroblasts grown in Spinner culture.

Stock virus preparation. Pr-RSV was retracted four times essentially as described by Graf et al. (10). A large stock of the final clone was frozen in 10-ml portions at -85 C and served as the inoculum for each experiment. Stocks of other viruses were prepared and stored similarly.

Chicken embryo fibroblast culture techniques. Fertile Hyline White Leghorn eggs were obtained from a local hatchery and from an inbred flock of chickens maintained at North Carolina State University. Primary chicken embryo fibroblast cultures were prepared as described by Vogt (29) and pretested for overt congenital leukemia virus by the standard RIF test (21). The basic tissue culture medium, Ham F10 (12), was chosen since it is...
relatively simple, can be made in the laboratory with a minimum expenditure of time and reagents, and is also available from most commercial supply houses. All media were supplemented with beef embryo extract (29), streptomycin (50 μg/ml), penicillin (50 units/ml), mycostatin (10 units/ml), and tryptose phosphate broth (Difco). Dimethylsulfoxide was added at a final concentration of 1% to the medium of all virus-producing cells (31).

Growth medium (GM) consisted of the above ingredients plus 5% calf serum (Gibco) and was used for all nontransformed cells and for subconfluent transformed cells. Maintenance medium (MM) consisted of 1% calf serum and was used for confluent monolayers of transformed cells.

Roller culture bottles (1,410 cm² growth area, 490-mm long by 110-mm diameter, Bellco Glass, Inc., Vineland, N.J.) were cleaned after use by filling with warm water and adding approximately 20 ml of undiluted 7x detergent and Chlorox. The combined detergent-bleach decontaminated the bottle with respect to infectious virus and hydrolyzed cellular proteins. Bottles were washed in an automatic dishwasher and sterilized in a dry oven.

Virus concentration. Cells were removed from the supernatant fluid by centrifugation at 1,500 rpm for 15 min. Depending on the volume of fluid to be processed, virus was pelleted from supernatant fluids by centrifugation in one of the following: (i) up to 210 ml, Beckman SW27 rotor, 25,000 rpm, 60 min, (ii) up to 1,500 ml, Beckman 19 rotors, 18,000 rpm, 90 min, (iii) and up to 3,000 ml, Beckman J21 centrifuge with JA10 rotor, 8,000 rpm, 6 h. Pellets were resuspended in TE buffer (0.005 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.001 M ethylenediaminetetraacetic acid (EDTA), pH 8.6) with sonic treatment for 30 s at room temperature in an ultrasonic cleaner (model 7, Heat Systems, Inc. Plainview, N.Y.).

Ammonium sulfate concentration was performed as described by Duesberg et al. (8). An equal volume of neutral saturated ammonium sulfate was added dropwise to supernatant fluids with constant stirring at 4 C. After standing 1 to 2 h, flocculated material was pelleted by centrifugation at 1,500 rpm for 15 min. The pellet was transferred to a dialysis bag and dialyzed against TE buffer.

Polyethylene glycol (PEG) precipitation was performed essentially as described by McSharry and Benzinger (18). The NaCl concentration was adjusted, and solid PEG (mol wt 6,000, Gallard-Schlesinger) was added slowly with constant stirring. After the PEG had dissolved, the mixture was placed at 4 C overnight, after which it was centrifuged at 1,500 rpm for 15 min. The effect of salt concentration between 0.1 and 0.5 M and PEG concentration between 2 and 20% was tested. If pellets were absent and the supernatant fluids remained cloudy after centrifugation at 1,500 rpm for 15 min, the speed of centrifugation was increased until pellets were obtained. Pellets were resuspended in SB (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.2] 0.001 M EDTA). The effectiveness of precipitation was measured by titrating infectivity.

**Virus purification.** The following density gradient systems were evaluated. (i) For sucrose density gradients, sucrose was dissolved in TE buffer. Isosmotic Ficoll-sucrose gradients were prepared as described by Day et al. (7). (ii) Glycerol density gradients were TE at the top and undiluted glycerol at the bottom. (iii) Potassium tartrate density gradients were made with TE at the top and 68% (wt/vol) potassium tartrate dissolved in TE at the bottom. (iv) Sodium citrate density gradients were TE at the top and 60% (wt/vol) sodium citrate dissolved in TE at the bottom. (v) Cesium chloride solutions were made in TE, and the cesium chloride density gradients were 1.1 g/ml to 1.3 g/ml. All density gradients were linear.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis was performed in a slab-gel apparatus, and the gel mixture was as follows: 12.5% acrylamide, 0.03% bis-acrylamide, 0.1 M phosphate buffer (pH 7.2), 0.1% N,N,N',N'-tetramethylethylenediamine, 0.02 M EDTA, 5 M urea, and 0.1% sodium dodecyl sulfate (SDS). Polymerization was catalyzed with 0.8% ammonium persulfate. Protein concentrations were measured by the method of Lowry et al. (15). Virus samples of 40 µg were pelleted, dissolved in 8 M urea, 2% SDS, and 1% 2-mercaptoethanol, heated at 100 C for 2 min, and subjected to electrophoresis at 50 mA and 35 V for 18 h. The gels were then stained for 2 h with 0.2% Coomassie blue in methanol-acetic acid-water (50:10:40) and destained with 7% acetic acid. They were traced using a Gilford spectrophotometer equipped with a linear transport device.

**RESULTS**

**Large-scale production of virus.** Virus-producing cells were grown in petri dishes, in Spinner culture, and in roller culture. Since cells did not grow in Spinner culture (see below), and large-scale growth of virus in petri dishes was laborious, roller culture was investigated more thoroughly.

Since transformed cells grew more rapidly in roller cultures than nontransformed ones, cells were infected and transformed in petri dishes before transfer to roller culture bottles. Primary chicken embryo fibroblasts were seeded into 100-mm plastic petri dishes (Falcon) at 5 × 10⁹ cells per dish and infected immediately at a multiplicity of 0.01 focus-forming units per cell. Polybrene was added to the culture medium at a concentration of 2 µg/ml (28) and was left in the medium until the first transfer. After 3 to 4 days, cells were trypsinized and seeded into 150-mm petri dishes at the rate of 15 × 10⁴ cells per dish; four days later all cells were transformed. The cells were then trypsinized again, and seeded at the rate of 10⁴ cells per bottle into 1,400 cm² roller culture bottles containing 100 ml of GM. Bottles were rotated in a roller apparatus at 6 rph. After 24 h the
medium was replaced with 200 ml of GM; 48 h later, daily feeding with 200 ml of medium was started. Cells were fed with GM until confluency (4–5 days after seeding) whereupon the serum concentration was lowered to 1% (MM) for maintenance. Harvesting of virus was then begun.

Once confluency had been attained in roller culture bottles, the cells could be trypsinized and transferred. This procedure quickly increased the total number of roller culture bottles in production, since one confluent roller bottle could be seeded into 10 new ones. The trypsinization procedure for roller culture bottles was as follows. The medium was poured off, and the cells were rinsed with 100 ml of warm Tris-buffered saline. Cells were trypsinized at room temperature with 100 ml of warm 0.05% trypsin (Schwarz/Mann, 1:300) while rotating by hand; cells were completely removed within 2 to 3 min. The trypsinized cells were placed into a flask containing 100 ml of 10% calf serum in Tris-buffered saline, counted, and seeded at the rate of 10^5 cells per bottle into fresh roller culture bottles containing 50 ml MM. After 24 h, the medium was replaced with GM, and the cells were fed daily thereafter.

Although transformed cells could be transferred, provided that they were trypsinized soon after they had become confluent, repeated passage of cells in this manner was not an effective means of long-term culture. After several passages, the monolayer acquired a glassy transparent appearance and failed to reach as high a cell density at confluency as freshly transformed cells. Another means of cell propagation was to use the cells present in the supernatant fluids of daily harvests. Roller culture bottle monolayers containing 10^4 cells per bottle often generated as many as 10^6 viable cells per day in the culture medium. Since these supernatant cells attached and grew with the same plating efficiency as trypsinized cells, one new roller culture bottle could be started daily from each established roller culture bottle.

Virus could be harvested continuously from roller culture bottles maintained for long periods of time without transfer. An analysis of virus production from three long-term roller culture bottles is shown in Fig. 1. Virus production remained constant during 32 weeks of culture. From 3 to 6 mg of purified virus was routinely recovered per liter of supernatant fluid every 24 h.

Large-scale production of avian tumor viruses by Spinner cultures of transformed cells (1) was unsuccessful. Cells failed to divide in Spinner culture despite frequent medium changes, increasing the calf serum concentration, increasing the concentration of vitamins and amino acids, and growing them at various cell densities. Cells transformed by Pr-RSV, RSV(RAV-1), RSV(RAV-2), RSV-SR-B, and MC29-B were all tested for their ability to grow in Spinner cultures; none would do so.

**Concentration of virus.** The optimal method for virus concentration was pelleting. A comparison of pelleting, ammonium sulfate precipitation, and PEG concentration is shown in Table 1. Pelleting consistently gave a higher recovery of infectious virus than the other two methods. Another factor which influenced the choice of concentration procedure was the behavior of the concentrated virus upon subsequent analysis by density gradient centrifugation. Direct comparison of virus preparations concentrated by pelleting, ammonium sulfate, and PEG precipitation indicated that pelleted virus consistently gave the highest yields of material banding in sucrose at a density of 1.16 g/ml.

**Purification of virus: use of the B XIV zonal rotor.** Large-scale virus purification was facilitated by the use of the B XIV zonal rotor. The purification of virus pelleted from 3 liters of supernatant fluid is shown in Fig. 2. Velocity sedimentation (Fig. 2A) separated virus from material which remained at the top of the sucrose gradient. The material at the bottom of the gradient was probably cellular debris, since electrophoresis of this material on polyacrylamide gels indicated that very few virus specific polypeptides were present. The equilibrium sedimentation profile (Fig. 2B) indicated a homogeneous virus band clearly separated from minor contaminating bands. Up to 200 mg of virus could be purified in the zonal rotor per day.

Table 2 shows the recovery of virus from 29 liters of supernatant fluid. Ninety-three milligrams of virus was recovered, corresponding to 3.2 mg per liter of original material. The infectivity remained relatively constant through the first purification step (velocity density gradient sedimentation) but decreased after the second step (equilibrium density gradient centrifugation). This loss was usually encountered during the second density gradient centrifugation step, even if equilibrium density gradient centrifugation was performed first.

Analysis of the polypeptides associated with virus at various stages of purification on SDS polyacrylamide gels indicated that the number
FIG. 1. Analysis of the production of focus-forming virus from three long-term roller culture bottles. Cells transformed with Pr-RSV were seeded into roller culture bottles, supernatant fluids were collected and pooled daily. Samples were removed from the pooled supernatant fluid at weekly intervals, and focus-forming activity was determined on chicken embryo fibroblasts.

Table 1. Relative recovery of Pr-RSV FFU by three concentration methods

| Concentration method                        | % Recovery |
|---------------------------------------------|------------|
|                                             | Expt 1     | Expt 2 | Expt 3 |
| Precipitation with ammonium sulfate         | 32         | 8      | 26     |
| Precipitation with polyethylene glycol      | 12         | 20     | 32     |
| Pelleting by centrifugation at 25,000 rpm for 60 min | 50         | 100    | ND     |

*FFU, Focus-forming units.
*ND, Not done.

of polypeptides decreased with each stage of purification. It could be shown in this way that equilibrium density gradient centrifugation after velocity density gradient centrifugation was an essential step of purification since four minor bands were removed by equilibrium density gradient centrifugation. The polypeptide pattern of purified virus is shown in Fig. 3. Fourteen polypeptides were present, including four low-molecular-weight species which were the group-specific antigens (3, 5), and 10 minor bands which ranged in molecular weight from 28,000 to 110,000.

Purification using swinging bucket rotors. Similar conditions of purification were applied to smaller scale preparations which were especially useful for virus labeled with radioactive materials. Virus was pelleted from the supernatant fluid and resuspended in TE buffer at the rate of 1 ml per liter of supernatant fluid. This pellet was resuspended by sonic treatment for 30 s in an ultrasonic cleaner, and the resulting suspension was centrifuged at 5,000 rpm for 15 min. The pellet which contained primarily cellular material, as judged by polyacrylamide gel electrophoresis patterns,

FIG. 2. Velocity and equilibrium density gradient centrifugation of Pr-RSV using a B XIV zonal rotor. A. Virus from 3 liters of supernatant fluid was pelleted, suspended in TE buffer by brief sonic treatment, layered onto a 15 to 40% (wt/vol) sucrose density gradient with a 45% (wt/vol) sucrose cushion and a TE overlay, and centrifuged for 15 min at 45,000 rpm (ωt = 2.3 × 10^14). The gradient was displaced with a 55% (wt/vol) sucrose solution. Optical density at 260 nm was recorded with a Beckman DU spectrophotometer. The top of the gradient is at the right. B. The virus band (indicated by the bar) was diluted to 400 ml with TE and layered onto a 15 to 60% (wt/vol) sucrose density gradient with a 65% (wt/vol) sucrose cushion and a TE overlay, and centrifuged for 70 min at 45,000 rpm (ωt = 1.5 × 10^14). The gradient was displaced with a 70% (wt/vol) sucrose solution and monitored as in A. The total height of the recording is 2 optical density units.

Table 2. Recovery of virus during purification in the zonal rotor B XIV

| Component                                  | Protein (mg) | Focus-forming Units |
|--------------------------------------------|--------------|---------------------|
| Cell-free supernatant fluid (29 liters)    | ND           | 29 × 10^10          | 100     |
| Unpurified virus pellet                     | 416          | 12 × 10^10          | 41      |
| After velocity density gradient centrifugation | 212          | 12 × 10^10          | 41      |
| After equilibrium density gradient centrifugation: purified virus | 93           | 1.3 × 10^10         | 4.5     |

*ND, Not done.
Forty micrograms of virus was dissociated with urea, SDS, and 2-mercaptoethanol and applied to a 12.5% acrylamide gel in a slab unit (see Materials and Methods). After electrophoresis, the gel was traced in a Gilford spectrophotometer. The highest peak represents 0.5 optical density units. The polypeptides are numbered according to Cheung et al. (5).

was discarded. Supernatant fluid containing virus was layered onto a 5 to 20% (w/v) sucrose density gradient, and centrifuged for 10 min at 30,000 rpm in an SW41 rotor. The virus band located near the center of the gradient was harvested and diluted with an equal volume of TE buffer, layered onto a 15 to 60% (wt/vol) sucrose density gradient, and centrifuged in a SW41 rotor for 3 h at 36,000 rpm. The virus band again appeared near the center of the gradient; it was collected, diluted with 5 vol of TE buffer, and pelleted. The pelleted virus was resuspended in TE at a final concentration of 5 to 15 mg of virus protein per ml.

**Choice of buffer for purification.** The choice of buffer system as solvent for sucrose solutions and as the pellet resuspension medium was found to be important. The buffer employed for most of these studies was TE. A comparison of TE with SB revealed that the use of TE resulted in less aggregation. Centrifugation of virus purified by equilibrium density gradient centrifugation in velocity density gradients composed of TE caused a small peak of impurities (as judged by polyacrylamide gel electrophoresis and electron microscopy [16]) to separate out near the bottom of the gradient. Density gradients composed of sucrose dissolved in SB did not resolve this second peak nearly as well. Separation of this peak clearly emphasizes the need for both equilibrium and velocity density gradient centrifugation in the purification of RNA tumor viruses.

Virus purified by both equilibrium and velocity density gradient sedimentation using either TE or SB was examined in the electron microscope. Virus purified in the presence of SB showed considerable aggregation, whereas virus purified in TE was well dispersed. It is clearly important to maintain virus in mono-disperse suspension, since aggregation tends to trap contaminating cellular debris.

**Choice of the major component of density gradients.** Sucrose was compared with several other materials used to form density gradients, such as Ficoll, glycerol, sodium citrate, potassium tartrate, and cesium chloride. Sodium citrate, potassium tartrate, and cesium chloride all caused aggregation, as evidenced by the generation of flocules; large amounts of material at the top of these gradients indicated that some particle disruption may have occurred. When virus was sedimented in glycerol density gradients, two bands close together appeared near the bottom of the tube; both appeared to contain virus predominantly since no difference was found in the polypeptide pattern of material recovered from them. Ficoll gave good results, especially when used under isoosmotic conditions (7) in the presence of D$_2$O, but virus yields were lower in Ficoll than in sucrose, and a dense band near the origin of the gradient suggested some particle disruption.

**Storage of purified virus.** Storage conditions for purified virus were evaluated by determining to what extent label in glycoprotein, presumably part of the viral membrane (3), remained associated with virus particles. Virus was labeled with $^3$H-fucose as described in the legend of Fig. 4, purified, and stored in various media at either 4 or $-85\degree$C for various periods of time, and then analyzed in equilibrium density gradients to determine the amount of $^3$H-fucose still associated with the virus band. The results indicated that virus stored at $-85\degree$C in TE (Fig. 4) or SB (not shown) was almost completely disrupted. However, the addition of calf serum to a final concentration of 10% almost completely prevented disruption. The addition of insulin, glycerol, or sucrose had a stabilizing effect similar to that of calf serum. Virus could be stored in TE at 4 $\degree$C for up to 2 weeks without loss of glycoproteins (data not shown).

**Measurement of purified virus concentration.** Analysis of protein by the Lowry technique (15) was a satisfactory me-
and since one virus particle has been estimated to weigh $7.5 \times 10^{-15}$ mg (4), one OD$_{260}$ nm corresponds to $3.1 \times 10^{11}$ virus particles.

**DISCUSSION**

Large-scale production of virus. Roller culture of Pr-RSV transformed chicken embryo fibroblasts was the most satisfactory method for large-scale virus production. Culture in petri dishes was laborious, since about 20 petri dishes were required to achieve the same number of cells obtained from a single roller culture bottle. Furthermore, contamination was more frequent. Culture of transformed chicken embryo fibroblasts in Spinner culture was attempted but was unsuccessful. Long-term growth of cells in roller culture was attempted because previous experience with growth of transformed cells in petri dishes indicated that transfer was not required to maintain cell viability and high virus titers. Transfer was unnecessary when Pr-RSV-transformed cells were grown in roller culture bottles. The long-term growth of these cells can possibly be attributed to the following. (i) The decrease in cell adhesiveness associated with transformation (6) caused large numbers of cells to be released into the supernatant fluid. Daily harvesting of supernatant fluid in the course of virus production provided a mechanism for removing excess cells from the roller culture. (ii) Changing the calf serum concentration from 5% (GM) to 1% (MM) upon confluency probably contributed to the balance of cell

![Graph](image-url)
growth and removal by slowing cell growth without damage to the transformed cells, which in any case required less serum (17, 27).

Transformed cells have been grown in roller culture bottles for 43 weeks. These cells produce virus, produce cells into the supernatant fluid at the rate of 1.5 × 10^7 cells per bottle per day, and remain healthy. The possibility that these cells have become an established avian cell line is currently being tested by establishing the cloning efficiency, testing their ability to grow in Spinner culture, testing their ability to grow in soft agar, and other relevant parameters.

Subgroup C avian tumor viruses may be the best for large-scale production, since they are formed in large amounts and cells transformed with them can be cultivated for long periods of time. Members of other avian subgroups have the following disadvantages. Subgroup A viruses remain attached to cells after budding, resulting in relatively low titers in the supernatant (unpublished data). Subgroups B and D are relatively cytopathic, and long-term cultivation is difficult (unpublished data); the cytopathic nature of these subgroups is reflected in the recent development of plaque assays for these subgroups (11, 14). Subgroup E viruses grow only to low titers in tissue culture.

**Virus concentration and purification.** Optimal virus concentration was achieved by pelleting virus from the supernatant fluid. Precipitation with polyethylene glycol (18) and ammonium sulfate (8) gave lower recovery of infectious virus and less virus banding in sucrose density gradients at 1.16 g/ml. Various methods of pelleting were similar in recovery rates. The largest volume of supernatant fluid was accommodated in the JA10 rotor of the Beckman J21 centrifuge, which processed up to 9 liters of supernatant per day.

Virus was purified by banding in successive sucrose density gradients constructed to separate particles on the basis of both size and density. This was essential since virus, banded only in either velocity density gradients or equilibrium density gradients, contained significant contamination as determined by polypeptide composition. TE buffer was chosen for dissolving sucrose and for resuspension of virus pellets because purified cellular membrane preparations (32) are stable in it. It gave far better separation from nonviral material than a buffer containing 0.1 M NaCl at pH 7.2 which also caused the virus to aggregate.

Storage of highly purified virus preparations at 4 C was satisfactory for up to 2 weeks. However, storage for longer periods of time necessitated freezing at −85 C and required the addition of stabilizing materials, such as calf serum, insulin, sucrose, or glycerol. The importance of such material is emphasized by the fact that purified virus was more susceptible to physical damage than was unpurified virus, since both sonic treatment and freezing caused disruption of purified virus and had little effect on unpurified virus.

Infectivity of virus remained relatively constant through the first density gradient but was reduced after the second. A similar loss of infectivity during a second purification step was noted by Spear and Roizman (25) for herpesvirus. Since the loss of Pr-RSV infectivity was encountered regardless of whether velocity or equilibrium density gradients were used, it seems likely that exposure to high sucrose concentrations alone cannot cause a large loss in infectivity, since virus, at equilibrium, bands at 34% (wt/wt) sucrose, whereas, during velocity sedimentation, virus often bands at 10% (wt/wt) sucrose. One possible source of infectivity loss is during the final concentration step, which is currently being done by pelleting. Since purified virus is more susceptible to damage by sonic treatment and freezing, it may also be damaged by the physical strains encountered during pelleting. We are currently investigating ways in which this damage can be eliminated, such as sedimentation of the purified virus onto a sucrose cushion, followed by dialysis against buffer.

**Measurement of purified virus concentration.** Finally, a comment may be made about the rapid method for estimation of purified virus concentration, based on optical density at 260 nm. This method has been found especially useful for poxvirus (13) and reovirus (24), since it permits rapid and accurate quantitation. A similar method based on turbidity at 540 nm has recently been described for AMV (26).

**ACKNOWLEDGMENTS**

L. H. Elrod provided skilled technical assistance in the development of the zonal purification techniques. Spinner culture experiments were performed in collaboration with Marie Stone. We gratefully acknowledge the assistance of R. B. Luftig for numerous electron microscope observations. We are indebted to R. Cook and D. Briggs of the North Carolina State University Department of Poultry Science for providing eggs from their inbred flocks. J. McAllister and K. Wojeski (Duke University), and G. Chandlee and J. Gresh (University Laboratories) rendered competent technical assistance. Finally, we wish to thank W. K. Joklik for innumerable helpful discussions and practical advice during the course of this study.

This investigation was supported by Public Health Service grant no. CA 12323 from The National Cancer Institute.
Health Sciences Advancement award no. 5 S04 RR 06148 and by Public Health Service contract no. PH 45-NCI-E-66-1135 awarded to University Laboratories, Inc., by the Special Virus Cancer Program.

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