Biophysical-Immunological Assay for Ribonucleic Acid Type C Viruses

J. OLPIN, S. OROSZLAN, AND R. V. GILDEN
Flow Laboratories, Inc., Rockville, Maryland 20852

Received for publication 14 March 1974

A biophysical and immunological method for characterization of ribonucleic acid type C virus suspensions is described. The method provides a relationship to the total viral mass concentration of the particle titer, the gs antigen titer, and the ultraviolet absorbance (268 nm) of 2% sodium dodecyl sulfate (SDS) digests. Data for murine, rat, feline, and hamster viruses are shown to be analogous within the test limitations. From these data, an assessment of the viral purity can be made, the structural integrity can be evaluated, an approximate molecular weight can be computed, and the mole ratio of gs antigen can be determined.

The properties of ribonucleic acid (RNA) tumor viruses have been the investigative concern of this laboratory for several years. The need for a convenient, reliable assay of viral titer is ever present. This communication describes such an assay showing the relationship between the number of virus particles per milliliter (estimated from counting particles observed in the electron microscope), the total viral mass (determined colorimetrically), the amount of gs antigen present (determined by radial immunodiffusion), and the ultraviolet absorbance measured at 268 nm (the absorbance peak of a 2% sodium dodecyl sulfate [SDS] digest).

Descriptions of routine assay methods for RNA type C viruses are generally lacking. The particle count method of Monroe and Brandt (12) was designed for generalized use, and utilized various viruses including one type C to generate a calibration curve giving a titer in particles per milliliter as a function of the number seen in the electron microscope grid opening. Particle count methods encounter certain difficulties when applied to type C viruses, all involving recognition of the virus. First of all, the virions are not particularly stable. Secondly, although referred to as a spherical virus, they do not have a distinct geometric configuration which is easily identifiable. Thirdly, the small amount of subcellular and subviral debris carried through the purification process often resembles virus, thus making the validity of the count questionable.

To minimize these difficulties, preparations can be assayed (i) before time or freezing disrupts the structure, (ii) at low pH (4.0 to 4.5) which induces tail-like formations on the virus thus enhancing their recognition in the microscope (21), and (iii) using only the peak fraction of the isolated viral bands, thus insuring a high virus/debris ratio.

MATERIALS AND METHODS

Viruses: murine leukemia virus. Several strains of murine leukemia virus were used in these studies. Rauscher murine leukemia virus was grown in monolayer cultures of chronically infected mouse (BALB/c) bone marrow, JLSV-9 cells (27). The virus-shedding cell line was obtained from Electro-Nucleonics Laboratories, Inc., Bethesda, Md. AKR virus-producing cells were kindly provided by Janet Hartley (National Cancer Institute, Bethesda, Md.). The La Puente isolate (1504E) of the wild mouse leukemia virus was obtained from Earle Officer (University of Southern California, Los Angeles, Calif.) (13; M. B. Gardner, J. E. Officer, R. W. Rongey, H. P. Charman, J. W. Hartley, J. D. Estes, and R. J. Huebner, Bibl. Haematol., in press) and was grown in monolayer cultures. New Zealand black mouse type C virus was grown in suspension cultures established from a fibrosarcoma, SCRF 60A, at Scripps Clinic, La Jolla, Calif. (8). AT-124 mouse virus grown in human rhabdomyosarcoma cells was propagated as described by Todaro et al. (24). Moloney mouse sarcoma virus was produced in a rat tissue culture cell line, 78A1 (1), obtained through the courtesy of Maurice Green (Inst. of Molecular Biology, St. Louis, Mo.).

Rat type C viruses. Two different strains of rat type C viruses, obtained from chronically infected tissue culture cell lines were used. The Rauscher pseudotype of the Moloney murine sarcoma virus was obtained from the MSB-1 cell line (23) derived from a tumor induced by Moloney murine sarcoma virus in a female rat of the Brown Norway strain, as previously described and characterized (14). The other strain was grown in the RPL cell line (6, 25), derived from Lewis
rat embryo fibroblasts exposed to Rous sarcoma virus. It was kindly provided by Vaclav Klement (University of Southern California, Los Angeles, Calif.).

**Feline type C viruses.** Feline leukemia virus (Theilen strain) was obtained from a chronically infected cat lymphocytic cell suspension culture (22). RD-114, an endogenous cat virus was the isolate from the human rhabdomyosarcoma cell line of McAllister et al. (11).

All monolayer cultures were supplemented by 10% fetal bovine serum, and suspension cultures were supplemented by 20% fetal bovine serum.

**Virus purification.** Tissue culture fluids were clarified by filtration through a membrane filter (Millipore Corp., 1.2 μm pore size). The virus was concentrated by continuous flow centrifugation with isopycnic banding in tris(hydroxymethyl)aminomethane (Tris)-buffered (0.01 M, pH 7.4) sucrose gradients (20 to 60% wt/wt). Fluid volumes of 20 to 30 liters were collected at flow rates of 4.0 to 4.5 liters/h through a CF-32 continuous flow rotor (holding a 25 to 50% sucrose linear gradient) operated at 32,000 rpm (102,000 × g) by an L-350 ultracentrifuge (Spinco). Larger volumes (50 to 100 liters) were collected at flow rates of 13 liters/h through the K-6 continuous flow rotor (holding a 25 to 60% sucrose linear gradient) operating at 35,000 rpm (90,000 × g) in the model K Mark II ultracentrifuge (Electro-Nucleonics). The concentrated virus was thereafter diluted and rebanded a second time using either Ti-14 (at 172,000 × g), Ti-15 (at 102,000 × g), or JCF-Z (at 40,000 × g) zonal rotors (Spinco) operating for 1, 2, or 3 h, respectively. A portion of the fraction having the maximum absorbance (monitored at 280 nm) was utilized for the assays described here.

**Particle counting.** A volume of 0.1 ml of each virus dilution was added to 0.1 ml of 0.1% bovine serum albumin, and 0.8 ml of 0.05 M sodium citrate, (pH 4.2). A drop of this mixture was applied to a Formvar-covered, carbon-coated 400-mesh grid for 5 min after which the drop was removed by aspiration onto filter paper. Three successive drops of 1% phosphotungstic acid, pH 4.2, were then applied, each remaining 1 min before removal by filter paper. The average number of viruses present per grid opening was calculated from five separate determinations and the titer was estimated from the calibration plot of Fig. 1. The calibration was prepared using solutions of polystyrene latex spheres of known concentration (Dow Chemical, run no. LS-1045E, diameter = 0.176 μm). Application of the polystyrene suspensions to electron microscope grids was performed in the same manner as the viral preparations. The number of latex spheres in each of 25 grid openings was determined for each concentration prepared. The mean and standard deviation were computed for each set of 25 and plotted in Fig. 1A.

**Determination of viral mass concentration.** Virus concentrations in milligrams of virus per milliliter were determined by a combined colorimetric/dry weight analysis. A volume of 1 to 2 ml of virus was exhaustively dialyzed versus running deionized water. The viral sample was appropriately divided, 0.5 to 1.0 ml being lyophilized in duplicate in tared ampoules, while the remainder was assayed in triplicate by a modification of the Lowry (9) method. This modification uses the reagents as described by Shatkin (20), but in 5× amounts while keeping the total sample weight concentration less than 250 μg. Absorbances were read in a Zeiss M4QIII or Beckman DU spectrophotometer using a 1-cm or 5-cm path length cuvette. All samples were read against a water blank.

**Ultraviolet absorbance measurements.** Virus suspensions were solubilized by the addition of SDS (Sequenal Grade, Pierce Chemical) at a ratio of 4 parts viral suspension to 1 part 10% (wt/vol) SDS. The absorbance envelope from 250 to 300 nm was measured using a similarly prepared water = 10% SDS (4:1) sample as blank reference.

---

**FIG. 1.** (A) Particle count data showing the relationship of virus concentration in particles per milliliter to the number of particles seen per grid opening. These data were compiled with polystyrene latex sphere (PLS) suspensions of known concentration and assuming that adhesion to the grid coatings is the same for both PLS and virus. (B) Colorimetric data showing the absorbance developed at 660 nm by the Folin phenol reagent, by the method described in the text, on known weights of whole virus. The relationship for the data is 7.7 × 109(A660) = micrograms of virus (S = ± 13 μg). For comparison, the color development of bovine serum albumin to the Folin phenol reagent is shown as a dashed line.
Quantitative determination of gs antigen by radial immunodiffusion. Radial immunodiffusion (10) was carried out in a Gelman immunodiffusion system following previously described techniques with minor modifications (4, 7). To prepare antiserum-agarose plates, portions of antiserum were mixed with warm agarose (0.8%) dissolved in Tris buffer, pH 7.4, ionic strength 0.15, containing 9.3 g of Tris 2-amino-2-(hydroxymethyl)-1,3-propanediol, 74 ml of 1 N HCl, and 7.0 g of NaCl made up to 1 liter with distilled water (3). Merthiolate (1:10,000) was added as a preservative. Six glass slides (76 by 26 mm) were placed in a polystyrene frame, and 11.5 ml of warm antiserum-agarose solution per frame section (3 slides) was spread on the slides to obtain a uniform layer. Four wells (3 mm diameter) were punched in each slide in the gel layer and filled with 10 μl of antigen solution in Tris buffer. After incubation at room temperature for 48 h, the diameter of the precipitin ring around each well was measured with a transparent ruler.

Antisera. Specific antiserum to the major group-specific antigen (p30) of mammalian type C viruses were prepared by immunizing goats with proteins purified by isoelectric focusing (14-17). Antiserum to whole bovine serum raised in rabbits was purchased from Kallestad Laboratories (Minneapolis, Minn.).

RESULTS

Shown in Fig. 1 are: (A) the polystyrene latex sphere particle count data from which the viral titer is estimated, and (B) the colorimetric data from which the viral mass is estimated. The response curve of bovine serum albumin to the modified Lowry technique is also shown in Fig. 1B. Regression analysis indicates that the estimate of particle titer from the count (Fig. 1A) is accurate to within ±0.5 log at dilutions as low as 5 × 10^4 per ml with accuracy increasing with concentration. The limits of detectability for counting particles, which is that dilution where one particle appears in the grid opening, is about 3 × 10^6 per ml. The absorbance relationship (Fig. 1B) fits the linear regression line: 7.7 × 10^2 Aₜₐₜ = micrograms of virus (SD = ±13 g).

The radial immunodiffusion calibration curve, from which the gs titer is measured, is shown in Fig. 2, along with a typical radial immunoprecipitin pattern for several dilutions of anti-murine leukemia virus gs. The gs titer is estimated with precision in the range of 6 to 100 μg/ml which corresponds to a diameter of the immunoprecipitin ring of 6 to 14 mm.

The relationships of the particle titer, gs titer, and absorbance at 268 nm of a 2% SDS digest, to the viral mass are shown in Fig. 3. Regression analysis of particle count versus mass data (Fig. 3A) shows a standard error of estimate of particle concentration of ±300 particles per ml. The double log plot is curvilinear due to a spatial underemphasis of a negative y intercept.

![Graph showing the relation between the quantity of isoelectric focus purified gs antigen and ring diameter. Inset represents duplicate immunodiffusion patterns obtained with increasing concentrations of gs antigen, 6.125, 12.25, 25, and 50 μg/ml, respectively.](http://aem.asm.org/)
Absorbance spectra of SDS digests of type C particles have a maximum at 268 nm (Fig. 3B), and at 268 nm has a one-to-one relationship to viral concentration in milligrams per milliliter. The gs titer data (Fig. 3C) reveal that 16 ± 1% of the total viral mass is comprised of this antigen. Assuming 60 to 65% of the virion to be protein (18, 19), the gs protein/viral protein ratio would be 27 to 25%, respectively. Radial immunodiffusion studies with anti-fetal bovine serum gave no response to any viral preparation, except one which indicated ≤5% fetal bovine serum of the total mass concentration.

DISCUSSION

The data shown in Fig. 1B and 3A are the results of three to six determinations on each viral line. Those of Fig. 3B represent one to three determinations for each viral line. Due to the statistical uniformity of the data it was not felt necessary to delineate the data obtained for each viral type by varying symbols. For example, the 60 points of Fig. 1B have a linear correlation coefficient of 0.97 and a standard error equal to 0.016 absorbance units. This is similar to the precision of the test itself when performed on an arbitrary standard such as bovine serum albumin. The linear correlation of the data in Fig. 3A (=0.84) is not nearly as good as above, but the standard error is ≤0.3 log which is sufficiently precise to enable one to give a quantitative description of the viral suspensions. Additionally, there appear to be no obvious differences in the data that would differentiate one virus from another.

The development of the modification of the Lowry method resulted from pronounced non-linear color responses of viral protein when standard procedures (9) were used (Fig. 4). While non-linearity could be tolerated if the standard was based on virus itself, it is com-

![Graphs](http://aem.asm.org/)

**Fig. 3.** (A) The relationship of the viral titer to the viral mass as determined by procedures summarized in Fig. 1. The line of best fit appears curvilinear due to the plotting method (see text). The standard error is equal to ±260 particles/ml. (B) Absorbance at 268 nm of 2% SDS viral digests. The data show an approximate 1:1 relationship and afford a rapid, easy method of obtaining viral titer. The inset shows a typical ultraviolet absorbance spectrum of an SDS digest with the abscissa giving wave length in nanometers and absorbance values given by the ordinate at left. (C) Relationship of gs titer to viral mass titer as determined by the radial immunodiffusion method described in Fig. 2, and the Folin phenol method as described in Fig. 1B. The data indicate that 16 ± 1% of the viral mass is gs antigen. This is equivalent to about 26% of the total viral protein assuming the type C virion to be 60 to 65% protein.
Fig. 4. Comparative viral mass determinations on arbitrary volumes of a marine leukemia virus preparation. The apparent concentration, determined in micrograms per milliliter for each volume assayed is shown for the standard Folin Lowry (9) method (■) and the modified method described in the text (○). The standard method shows a significant inhibition as the volume tested is increased.

Fig. 4. Comparative viral mass determinations on arbitrary volumes of a marine leukemia virus preparation. The apparent concentration, determined in micrograms per milliliter for each volume assayed is shown for the standard Folin Lowry (9) method (■) and the modified method described in the text (○). The standard method shows a significant inhibition as the volume tested is increased.

Completely ambiguous when interpreted with bovine serum albumin as a standard. Since the new standard was mandatory, the modification to a linear response was an outgrowth of studies similar to those of Hartree (5), but simpler in technique. The standard curve of Fig. 1B is based on lyophilized weights instead of dry weights. Hence, an error of 10 to 15% in accuracy is expected due to bound water of lyophilized samples. Although the absorbance values are low with this procedure, they are very reproducible and as precise as indicated previously. If greater absorbances are desired, longer cuvette path lengths allow one to read in a more accurate spectrophotometer range without sacrificing linearity up to about 250 μg of virus.

Previous estimates have indicated that 1 mg of virus represents about 10¹² particles (2, 26), giving a virion molecular weight of about 6 × 10³. Since these estimates are based on the Lowry method (9) it should be noted that, given equal masses, bovine serum albumin gives a Folin phenol color development approximately 2.3 × that of type C virus (Fig. 1B). Therefore, it is to be expected that similar determinations of particle/mass ratios based on a viral standard would give enhanced values. Indeed, Fig. 3A shows a value of 1.8 mg/10¹² particles. This ratio leads to calculated molecular weight of approximately 10⁹. Although this value may be slightly inflated due to what intrinsic impurities remain, it is more accurate than when determined against a foreign standard such as bovine serum albumin.

The absorbance envelope of 2% SDS digested virus generally peaks at 268 nm (Fig. 3B), although some variations occur in shape. The peak absorbance value is equivalent to the mass concentration within ±5% and provides an easy, rapid measurement of viral content.

Comparisons of viral mass with particle concentration (using Fig. 3A) in normal production runs enable one to judge the relative amount of nonviral protein. For example, tests on arbitrary fractions though a continuous flow banded virus preparation reveal depressed count/mass ratios throughout the band indicating the inclusion of nonviral contaminants, whereas tests on arbitrary fractions of the rate zonal second banding show ratios in the range given in Fig. 3A. Repeated centrifugation does not there after enhance the count/mass ratio. Additionally, since the development of the radial-immunodiffusion assay for use with the RNA tumor viruses produced in this laboratory, 28 different viral preparations have been produced, all of which were analyzed by this method. Twenty-seven gave no detectable ring due to free gs antigen when tested as prepared. However, when treated with 0.1% Triton X-100 detergent, all gave a positive immunoprecipitin response indicating a gs content concomitant with that expected from a previous mass determination by the modified Lowry method. The 28th sample showed a native immunoprecipitin response to gs antisera of about 0.1 the level obtained after detergent release. Thus, we infer that 27 of the 28 production lots consisted of nearly 100% intact virus and the other contained approximately 90% intact particles. Thus, the threefold assay involving particle counting, mass determination, and radial immunodiffusion gives an estimate of viral content, purity, and structural integrity with greater precision than previously available.

ACKNOWLEDGMENTS
We thank David Bova, Carl Hebron, James Nupp, and William Clayget for their technical assistance. This work was supported by Public Health Service Contract NO1-CP-3-3247 of the Virus Cancer Program of the National Cancer Institute.

LITERATURE CITED
1. Bernard, C., M. Boiron, and J. Lasneret, 1967. Transformation et infection chronique de cellules embryonnaires de rat par le virus du sarcome de Moloney. C. R. Acad. Sci. 264:2120–2133.
2. Bonar, R. A., and J. W. Beard, 1959. Virus of avian myeloblastosis. XII. Chemical constitution. J. Nat. Cancer Inst. 23:183-197.

3. Crowle, A. J. 1961. Immunodiffusion, p. 302. Academic Press, Inc., New York.

4. Fahey, J. L., and E. M. McKelvey, 1965. Quantitative determination of serum immunoglobulins in antibody plates. J. Immunol. 94:84-90.

5. Hartree, E. F. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48:422-427.

6. Klement, V., M. O. Nicolson, W. Nelson-Rees, R. V. Gilden, S. Oroszlan, R. W. Rongey, and M. D. Gardner. 1973. Spontaneous production of a C-type RNA virus in rat tissue culture lines. Int. J. Cancer. 12:654-666.

7. Lanzerotti, R. H., and P. M. Gullino. 1972. Immunochemical quantitation of enzymes using multispecific antisera. Anal. Biochem. 50:344-353.

8. Lerner, R. A., F. Jensen, S. J. Kennedy, K. J. Dixon, G. Des Roches, and U. Francke. 1972. Karyotypic, virologic, and immunologic analyses of two continuous lymphocyte lines established from New Zealand black mice: possible relationship of chromosomal mosaicism to autoimmunity. Proc. Nat. Acad. Sci. U.S.A. 69:2965-2969.

9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

10. Mancini, G. A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:255-254.

11. McAllister, R. M., M. Nicolson, M. D. Gardner, R. W. Rongey, S. Rasheed, P. S. Sarma, R. J. Huebner, M. Hatanaka, S. Oroszlan, R. V. Gilden, A. Kabigting, and L. Vernon. C-type virus released from cultured human rhabdomyosarcoma cells. 1972. Nature N. Biol. 233:3-6.

12. Monroe, J. H., and P. M. Brandt. 1970. Rapid semiquantitative method for screening large numbers of virus samples by negative staining electron microscopy. Appl. Microbiol. 20:259-262.

13. Officer, J. E., N. Tescon, J. D. Eates, E. Fontanilla, R. W. Rongey, and M. B. Gardner. 1973. Isolation of a neurotropic type C virus. Science 181:945-947.

14. Oroszlan, S., D. Bova, R. J. Huebner, and R. V. Gilden. 1972. Major group-specific protein of rat type C viruses. J. Virol. 10:746-750.

15. Oroszlan, S., D. Bova, M. H. M. White, R. Toni, C. Foreman, and R. V. Gilden. 1972. Purification and immunological characterization of the major internal protein of the RD-114 virus. Proc. Nat. Acad. Sci. U.S.A. 69:1211-1215.

16. Oroszlan, S., C. Foreman, G. Kellogg, and R. V. Gilden. 1971. Antigenic differentiation of M-MSV(O) from mouse, hamster, and cat C-type viruses. Virology 43:722-724.

17. Oroszlan, S., R. J. Huebner, and R. V. Gilden. 1971. Species-specific and interspecific antigenic determinants associated with the structural protein of feline C-Type virus. Proc. Nat. Acad. Sci. U.S.A. 68:901-904.

18. Quigley, J. P., D. B. Rifkin, and E. Reich. 1971. Phospholipid composition of RSV, host cell membranes and other enveloped viruses. Virology 46:106-116.

19. Robinson, W. S., and P. H. Duesberg. 1968. The chemistry of the RNA tumor viruses. In H. Fraenkel-Conrat (ed.), Molecular basis of virology., Van Nostrand Reinhold Co., New York.

20. Shatkin, A. J. 1969. p. 231-237. Colorimetric reactions for DNA, RNA, and protein determinations. In Habel and N. Salzman, (ed.), Fundamental techniques in virology. Academic Press Inc., New York.

21. Stephens, R. K. Traul, G. Lowry, I. Zelljadt, and S. Mayyasi. 1972. Differential morphology of the RD virus from the human rhabdomyosarcoma, RD114B cell line demonstrated by negative staining electron microscopy. Nature N. Biol. 240:212-213.

22. Theilen, G. H., T. G. Kawakami, J. D. Rush, and R. J. Munn. 1969. Replication of cat leukemia virus in cell suspension cultures. Nature (London) 222:589-590.

23. Ting, R. C. 1968. Biological and serological properties of viral particles from a nonproducer rat neoplasms induced by murine sarcoma virus (Moloney). J. Virol. 2:865-868.

24. Todaro, G. J., P. Arnstein, W. P. Parks, E. H. Lennette, and R. J. Huebner. 1973. A type C virus in human rhabdomyosarcoma cells after inoculation into NIH Swiss mice treated with antithymocyte serum. Proc. Nat. Acad. Sci. U.S.A. 70:859-862.

25. Vesely, P., L. Donner, J. Cinatli, and V. Sovova. 1968. Interaction of Rous sarcoma virus with rat embryo fibroblasts of inbred Lewis strain in vitro. Folia Biol. (Prague) 14:457-465.

26. Vogt, P. 1965. Avian tumor viruses, p. 293-385. In K. M. Smith and M. A. Lauffer (ed.), Advances in virus research. Academic Press Inc., New York.

27. Wright, B. S., P. A. O’Brien, G. F. Shibley, S. A. Mayyasi, and J. C. Lasfargues. 1967. Infection of an established mouse bone marrow cell line (JLS-V9) with Rauscher and Moloney murine leukemia viruses. Cancer Res. 27:1672-1677.