JOURNAL OF VIROLOGY, Aug. 1977, p. 227-233

Vol. 23, No. 2
Printed in U.S.A.

Sizes and Concentrations of Several Type C Oncornaviruses and Bacteriophage T2 by the Resistive-Pulse Technique

RALPH W. DEBLOIS* AND ROY K. A. WESLEY[1]

General Electric Corporate Research and Development, Schenectady, New York 12301, and The John L. Smith Memorial for Cancer Research, Pfizer, Inc., Maywood, New Jersey 07607*

Received for publication 14 February 1977

Viruses above about 60 nm in diameter may be rapidly sized to a few nanometers in their natural hydrated state as they pass one by one through a single pore in a newly developed nanometer-particle analyzer based on the resistive-pulse technique of the Coulter Counter and the use of submicron-diameter pores made by the Nuclepore process. Size measurements for several type C oncornaviruses are: Rauscher murine leukemia, 122.3 ± 2 nm; simian sarcoma, 109.7 ± 3 nm; Mason-Pfizer monkey, 140.0 ± 2.5 nm; RD-114, 115 ± 5 nm; and feline leukemia, 127.4 ± 2 nm, relative to standard 109-nm latex spheres. The T2 bacteriophage has a volume of (5.10 ± 0.15) × 10^{-16} cm^3. Concentrations of viruses near 10^9 to 10^{11}/ml that are fairly clear of debris are routinely measurable in a few minutes to an accuracy near 15%. A lower practical count limit is near 5 × 10^7 viruses per ml.

Viruses are conventionally sized and counted by electron microscopy. Size measurements of a given virus have generally been quite variable, depending on preparative technique, leaving the original hydrated size uncertain. Concentration measurements involve several critical steps conducive to imprecision. We report here on a submicron-particle analyzer that can directly, accurately, and rapidly size and count viruses down to about 60 nm in diameter (4). The viruses are analyzed one by one as they pass in fluid suspension through a single pore. The "Nanopar" analyzer is based on the resistive-pulse technique of the Coulter Counter (W. H. Coulter, Natl. Electron. Conf. Proc. 12:1034, 1956) and the use of pores of submicron diameter made by the Nuclepore process (11). It represents about a 1,000-fold extension of the conventional Coulter Counter in volume sensitivity and thus opens up a new realm of particles to analysis by that technique.

The apparatus and techniques have been specifically developed beyond the initially reported state to count and size oncornaviruses grown at the John L. Smith Memorial for Cancer Research. We can now routinely measure concentrations near 10^9 to 10^{11} fairly clean and only moderately clumped viruses per ml in supernatant fluids from tissue culture growths or in dilutions of purified, concentrated viruses within a few minutes to an accuracy near 15% with either the analyzer or a simplified three-level counter. The analyzer has an experimental lower count limit near 5 × 10^7 viruses per ml because of thus far unavoidable background debris, although it is capable of determining concentrations near 10^5 virus-sized particles per ml. Viral sizes are readily measurable to within a few nanometers. The viruses are sized individually by volume in their natural hydrated state.

In this paper we present size measurements of several type C oncornaviruses (Rauscher murine leukemia, simian sarcoma, Mason-Pfizer monkey, RD-114, and feline leukemia viruses) and of T2 bacteriophage, and compare some concentration measurements with those obtained by electron microscopy. A further use of the analyzer in characterizing viruses by simultaneous size and electrophoretic mobility measurements is to be reported elsewhere (R. W. DeBlois, C. P. Bean, and K. A. Wesley, J. Colloid Interface Sci., in press).

MATERIALS AND METHODS

Apparatus. The heart of the Nanopar analyzer is a single pore of submicron diameter through which the particles to be analyzed pass one by one in fluid suspension. Each particle passing through the pore, through which an electric current is also flowing, displaces some of the conducting fluid while in the pore, producing a voltage pulse across the pore that is proportional in amplitude to the volume of the particle. These pulses are monitored on an oscilloscope, processed by an interface, and entered into a pulse-height analyzer to yield a histogram of number versus pulse height suitable for counting or sizing the particles. To demonstrate what is observed,

[1] Present address: Central Research, Pfizer, Inc., Groton, CT 06340.

we show in Fig. 1 a typical oscilloscope trace of the voltage pulses resulting from the consecutive passage of two Rauscher murine leukemia viruses through a 0.40-μm-diameter pore 3.8 μm long. There were 4.0 inches (ca. 10.2 cm) of water pressure, 0.608 V across the pore, and 33.8 nA passing through it. Because of space limitations, details of the apparatus and the procedures that have been developed to make a practicable analyzer are presented elsewhere (5; DeBlois et al., J. Colloid Interface Sci., in press).

Virus. Rauscher murine leukemia virus was grown in JLS-V5 cells (37) or JLS-V9 cells (28); simian sarcoma virus was grown in human lymphoblastoid cell line NC-37 (D. L. Larson, C. E. Garon, K. R. Harewood, J. S. Wolff, S. A. Mayyasi, and G. Schidlovsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V177, p. 224); Mason Pfizer monkey virus was grown in NC-37 or original monkey cells (24); and feline leukemia virus was grown in FL-74 cells (35). All cell cultures were maintained at 37°C in a tissue culture medium consisting of RPMI 1640 medium with 8 to 10% (15% for RD-114) heat-inactivated fetal calf serum. Bacteriophage T2 (American Type Culture Collection no. 11303 B_2) was obtained from Miles Laboratories, Inc., Elkhart, Ind.

Suspensions of oncornavirus were prepared either (i) directly by filtration of tissue culture medium from virus-infected cell cultures through 0.45- or 0.22-μm Millipore or 0.4- or 0.2-μm Nuclepore membrane filters, or (ii) by zonal sucrose density gradient centrifugation. In the latter case, the virus in the tissue culture medium was partially purified by continuous-flow centrifugation in a linear 15 to 55% isopycnic sucrose gradient, using a K-II ultracentrifuge. Further purification of the 25 to 45% banded virus was accomplished in an isopycnic sucrose gradient in a Spinco CF-32 continuous-flow rotor. The bands of virus were collected and centrifuged at 80,000 $\times$ g for 90 min in a Spinco 30 rotor. The resulting pellets were suspended in RPMI 1640 tissue culture medium and filtered as described above. Oncornaviruses were prepared for electron microscopy by the negative-staining method of Monroe and Brandt (25).

Procedure. Viruses in supernatant tissue culture medium, as well as Vortex-mixed resuspensions of centrifuged viruses in tissue culture or other protein-containing media, may generally be successfully analyzed when added after filtration at or below the diameter of the analyzing pore directly to the chambers of a cell containing the pore. In some cases, however, 1% or less of Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.) has been needed to relieve plugging of the pore. Although this nonionic surfactant does cause observable degradation of oncornavirus membranes in a matter of hours, it is a slow enough process to have negligible effect on size measurements made within minutes of its admixture, according to tests we have made in which suspensions of viruses with and without surfactant on opposite sides of an analyzing pore have been alternately analyzed by reversing the directions of flow. Since latex spheres clump when added at full strength to tissue culture medium, either Tween 80 is mixed with suspensions of virus before addition of the spheres or the suspension of spheres is first diluted in a 5% Tween 80 solution and the proper quantity of this mixture is added to the suspension of viruses.

Particle size measurements are made with the particles flowing from minus to plus electrical polarities at low pressure. Flow in the opposite direction is found to produce a distorted, upward-sloping pulse shape that we deduce is caused by a changing pore surface resistivity as the particle passes through, but that we do not specifically understand.

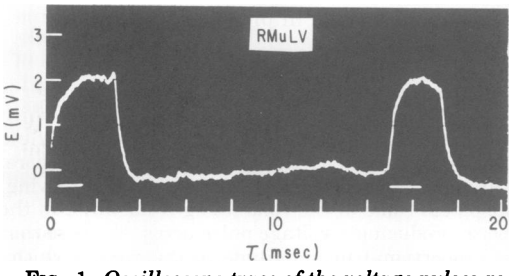

FIG. 1. *Oscilloscope trace of the voltage pulses resulting from the consecutive passage of two Rauscher murine leukemia viruses through a 0.40-μm-diameter pore. The pulse height is proportional to the volume of the virus.*

RESULTS

Virus sizes. In Fig. 2 we have a histogram of number versus pulse height for Rauscher murine leukemia virus. The virus is from a centrifuged production lot that had been resuspended in tissue culture medium and filtered with a 0.2-μm-pore Nuclepore membrane. The run was for 500 s at 12 inches (ca. 30 cm) of water pressure, with 1.000 V across a 0.33-μm-diameter pore 2.3 μm long. Calibration was made by subsequently adding to one cell chamber 1 μl of a mixture of 1 part standard 109-nm latex spheres (10% solids suspension, lot 2G3W, Dow Chemical Co., Indianapolis, Ind.) to 2 parts 5% Tween 80 in 0.1 N KCl, obtaining another histogram for the same conditions and subtracting the previous one, to leave the dashed-line histogram for the 109-nm spheres. The peak pulse heights of 34.5 and 50, with an instrumental zero correction of two units added to each, yield a diameter of 122.1 nm by use of equation 2 in the Appendix. The second histogram itself yielded a diameter of 122.3 nm, indicating negligible effect of the 1:30,000 concentration of surfactant on the size of the virus. Nine measurements on three different lots (the above resuspended pellet, a rediluted 100$\times$ concentrate, and a sample of supernatant fluid from a day-8 tissue culture growth) have given a peak diameter of 122.3 $\pm$ 2 nm. The standard deviation in the diameters of single viruses in a given distribution is about $\pm$7 nm.

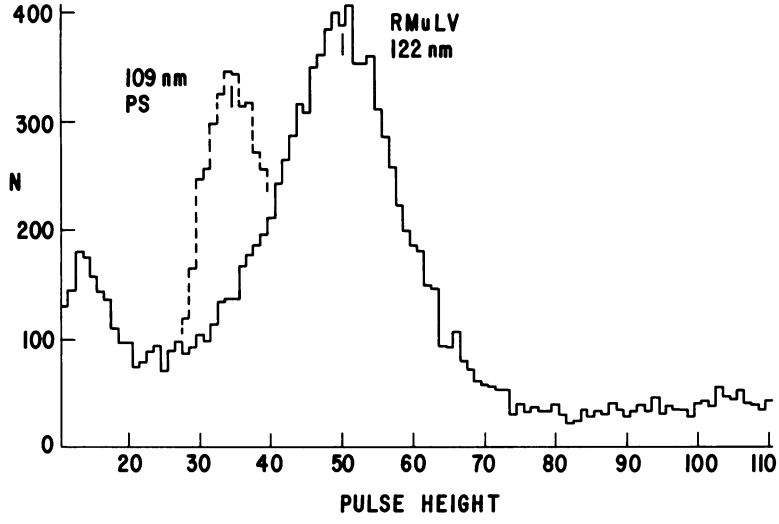

FIG. 2. *Size measurement histogram for Rauscher murine leukemia virus, showing a peak diameter of 122 nm relative to standard 109-nm latex spheres mixed subsequently with the virus.*

By similar procedures, we found a peak diameter of 109.7 nm for simian sarcoma virus from a production lot resuspended in tissue culture medium. Another resuspended lot gave a peak diameter of 112.7 nm with respect to 126-nm latex spheres (lot LS-025-4) that were mixed with it after dilution at 1:15 without any surfactant. However, previous (4) and current experiments indicate that the 126-nm spheres are actually about 121 nm in diameter if 109-nm spheres are taken as a standard. The 121-nm value yields 108.1 nm for the virus, 1.6 nm smaller than that given above.

Similar measurements on other oncornaviruses have yielded peak diameters of 140.0 ± 2.5 nm for Mason-Pfizer monkey virus for two different lots resuspended in tissue culture medium, with 0.5% Tween 80 and 109-nm latex spheres added just prior to sizing; 115 ± 5 for cat virus RD-114 from a tissue culture growth, with 1% Tween 80 added just prior to sizing; 134 ± 5 nm for feline leukemia virus (a production lot resuspended for 3 days in TNE [10 mM Tris-hydrochloride, 0.15 M NaCl, 10 mM EDTA]), with 0.5% Tween 80 added just prior to sizing; and 127.4 ± 2 nm for a fresh day-7 tissue culture growth of feline leukemia virus, with no surfactant for four runs (128.1 nm average) and 1% Tween 80 for three runs (126.5 nm average).

Figure 3 shows a histogram of number versus pulse height for a mixture of T2 bacteriophage and 109-nm-diameter latex spheres. The viruses and a diluted suspension of Tween 80-coated spheres were added to a 0.1 N glycine-0.17 N NaCl (pH 8.3) solution to a concentration near 3×10^{10} of each per ml. There were 1.500 V and 6 inches (ca. 15.2 cm) of water pressure across an approximately 330-nm-diameter pore. The run was for 304 s.

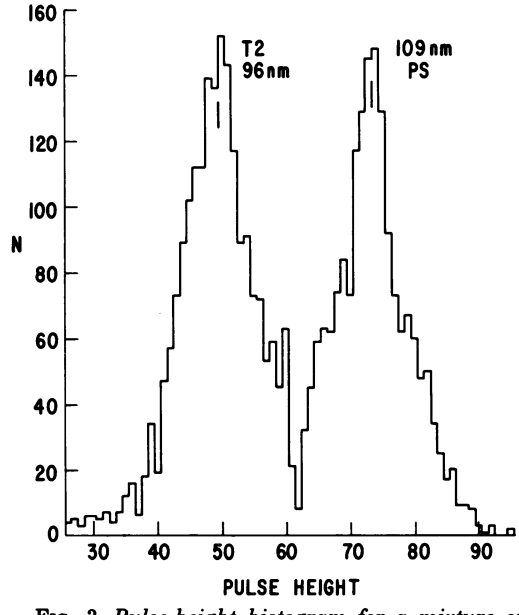

FIG. 3. *Pulse-height histogram for a mixture of T2 bacteriophages and 109-nm latex spheres. The calculated resistively equivalent diameter of 96 nm for the T2 virus leads, with shape corrections, to a volume of* 5.15×10^{-16} *cm*^{3}.

One finds a resistively equivalent diameter of 96.0 nm for the T2 virus with equation 2 for the observed peak pulse heights of 49.5 and 73.5, an instrumental zero correction of +1 pulse-height unit, and a pore diameter of 330 nm. This virus, however, has a polygonal head and a tail. If its shape is approximated as a

prolate ellipsoid with an axial ratio of 1.4:1 and the long axis is assumed to be parallel to the axis of the pore as it passes through (since no tumbling is observed experimentally, the virus is presumably so oriented by the electric field), then the form factor f_e is found to be $(1 - 0.26)^{-1}$, or 1.35 from equation 4, and equation 3 gives a volume for the virus of 5.15×10^{-16} cm^3, equivalent to that of a sphere 99.4 nm in diameter. A total of eight runs gave a resistively equivalent diameter of 95.7 ± 0.9 nm, a volume of $(5.10 \pm 0.15) \times 10^{-16}$ cm^3, and an equivalent-sphere diameter of 99.1 ± 1.0 nm.

Table 1 summarizes the size measurements. These measurements are with respect to standard 109-nm latex spheres. The absolute size of the standard spheres is in question. We find a value of 99 ± 5 nm by electron microscopy measurements against catalase crystals on the same grids, using Wrigley's (36) lattice-spacing value of 8.6 ± 0.2 nm for $1/2\ C_0$ (measurements by R. R. Russell at General Electric). Intensity fluctuation spectroscopy measurements (by E. E. Uzgiris at General Electric) also give a lower value of 101 ± 3 nm. Nevertheless, the standard value of 109 nm will be used for consistency and ease of comparison, pending further reliable measurements.

Particle counting. Concentrations of viruses that are fairly clear of debris and are only moderately clumped have been routinely measured in a few minutes in the range of 10^9 to 10^{10}/ml either (i) by count comparison (equation 8), in which a known number of 234-nm latex spheres are added to the suspension and the counts of viruses and spheres are obtained by integration over appropriate regions of the resulting histogram, with proper weighting for multiplets, or (ii) by simply measuring the flow rates of the viruses and using hydrodynamic equation 7. Determinations of concentration by the two methods have generally been consistent within about 15% (5). Corresponding measurements of concentrations in the above-mentioned range by electron microscopy have generally been several times lower, due partly to the presence of virus-sized debris that the Nanopar analyzer does not distinguish from virus, but more, we judge, from procedural loss of virus in the method of Monroe and Brandt (25).

An example of the determination of concentrations by the Nanopar analyzer and by electron microscopy is presented in Fig. 4. A centrifuged production lot of Rauscher murine leukemia virus was initially resuspended 1:10 in RPMI 1640 tissue culture medium, filtered at 0.45 μm, and subsequently further diluted 10- and 100-fold in 0.1-μm-pore-filtered medium. The polarity-averaged analyzer counts of 1.66×10^{10}, 1.76×10^9, and 1.55×10^8/ml for the respective dilutions (○ in the log-log plot) were calculated by using hydrodynamic equation 7. The 1:10 dilution, for example, gave a polarity-averaged flow rate of 19.9/s for two 500-s runs at 23.5°C and 10 inches (ca. 25 cm) of water pressure for a pore of resistance 12.27 MΩ in 0.1 N KCl at 19.4°C from a batch of 0.45-μm-diameter pores about 2 μm long. With a viscosity of 1.00 cP at 22°C and a rate corrected to 19.3/s at that temperature, the data yield the above concentration of 1.66×10^{10}/ml. The above concentrations are net results obtained after subtraction of runs for a 10^4 dilution, for which the histograms showed no discernible virus peaks. Apparently the 0.1-μm-pore-filtered medium still contained some debris.

TABLE 1. *Diameters of several oncornaviruses and volume of the T2 bacteriophage as determined by the resistive-pulse technique*

Determination	Diameter (nm)
Virus [a]	
RMuLV	122.3 ± 2
SSV	109.7 ± 3
M-PMV	140.0 ± 2.5
FeLV	127.4 ± 2
RD-114	115 ± 5
Phage	
T2	$(5.10 \pm 0.15) \times 10^{-16}$ cm^3

[a] RMuLV, Rauscher murine leukemia virus; SSV, simian sarcoma virus; M-PMV, Mason-Pfizer monkey virus; FeLV, feline leukemia virus.

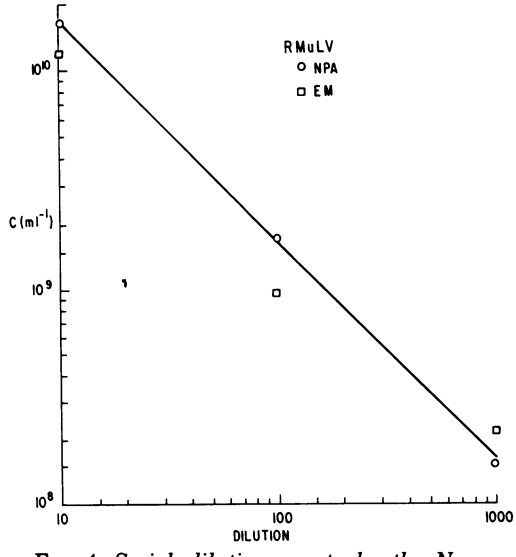

FIG. 4. *Serial dilution counts by the Nanopar analyzer* (○) *and electron microscopy* (□) *for resuspensions of a production lot of Rauscher murine leukemia virus, with a 45° line fitted to the analyzer data.*

A simultaneous run with a three-level counter that was set to count single viruses, doublets, and higher multiplets on the three counters gave concentrations of 1.77×10^{10}, 1.74×10^{9}, and 1.57×10^{8}/ml, respectively. A count comparison at one polarity only with 234-nm latex spheres on another 1:10 dilution of the concentrate gave 1.54×10^{10}/ml. Counts by electron microscopy were 1.12×10^{10}, 0.97×10^{9}, and 2.2×10^{8}/ml for the respective dilutions (□ in the plot).

DISCUSSION

The Nanopar analyzer demonstrates a new degree of precision and ease in this report in measuring the sizes of viruses. Moreover, the viruses are sized in their natural state—suspended in a fluid. This procedure avoids the distortions often involved in preparation for electron microscopy (23, 26, 31). The Nanopar analyzer may thus be used to make standard size measurements of viruses, as well as to establish what preparative techniques for electron microscopy best preserve the original viral structure. Intensity fluctuation spectroscopy (1, 14, 15, 28–30) can also size viruses suspended in a fluid and should be of similar accuracy if the suspension is pure and monodisperse. However, this technique measures a mean size of a suspension of particles, unless there are discrete components differing greatly in size, and would thus encounter more trouble with debris and polydispersion. It also cannot measure particle size distributions or resolve two close-lying components.

Viral concentrations near 10^{9} to 10^{11}/ml can be routinely measured in a few minutes with the Nanopar analyzer if the viral suspension is fairly clear of clumps and debris. The directness of the determination leads generally to greater accuracy than by negative-stain electron microscopy, as judged by latex-sphere comparison counts, although occasional observations by electron microscopy are needed to correct for virus-sized debris.

Rauscher murine leukemia virus, which we measure as 122.3 ± 2 nm in diameter, has been reported over the range of 85 to 150 nm as measured by electron microscopy (6–8, 10, 21, 22, 26, 31, 39), and as 145 ± 7 nm by intensity fluctuation spectroscopy (29). This last value might be compatible with ours if spikes of reported length 8 nm were presented on the envelopes (10), since they would make the virus appear larger hydrodynamically then resistively (15).

Feline leukemia virus has diameters by electron microscopy measurements of 100 to 115 nm (17, 19, 20, 33), and 120 to 140 nm if envelope spikes are included (9), compared with our 127.4 ± 2 nm for a fresh tissue culture growth and 134 ± 5 nm for virus suspended for 3 days in TNE. The latter value reveals a medium-dependent size, indicating that tissue culture medium might best be used as standard. Intensity fluctuation spectroscopy on virus from the same FL-74 cell line gives a diameter of 161 ± 5 nm (30). Envelope spikes probably partly account for this high value.

RD-114 virus has been measured by electron microscopy as approximately 100 nm in diameter (24), somewhat smaller than our measurement of 115 ± 5 nm. Mason-Pfizer monkey virus has been measured at about 110 nm (2, 18), compared with our 140.0 ± 2.5 nm, and simian sarcoma virus has been measured as 80 to 110 nm in diameter (13, 34), compared with our 109.7 ± 3 nm.

Our volume measurement of 5.10×10^{-16} cm^3 for the T2 bacteriophage (B_2 variety) is close to the 5.4×10^{-16} cm^3 we calculate from Gordon's measurements by electron microscopy on the T2L variety (12). This variety has been of special interest since the discovery by Hook et al. (16) of two distinct forms having sedimentation constants of about 1,000S and 700S, respectively. The difference appears to arise from the retraction or extension of tail fibers under different conditions of temperature and pH (12), rather than from earlier-reported changes in head size (3). Our volume measurement and measurements made showing only 1% change in volume under appropriately different conditions of pH support the differing tail-fiber configurations as the cause for the difference in sedimentation constants, but measurements specifically with the T2L variety would be needed to confirm it.

APPENDIX

Equations. The amplitude, ΔE, of the voltage pulse produced by a spherical particle of diameter d passing through a pore of diameter D is given approximately by

$$\Delta E \doteq \frac{Ed^3}{LD^2(1+\alpha)[1-0.8(d/D)^3]} \quad (1)$$

where E is the voltage across the pore, L is the effective resistive length of the pore (the geometric length plus $0.8\,D$), and α is the ratio of the resistance of the pore to the external resistance of the circuit.

The diameters of a mixture of two sets of spheres are compared through the equation

$$d \doteq d_0\left(\frac{\Delta E}{\Delta E_0}\right)^{1/3} \cdot \left[1+0.8\left(\frac{d_0}{D}\right)^3\left(\frac{\Delta E}{\Delta E_0}-1\right)\right]^{-1/3} \quad (2)$$

where d and d_0 are unknown and known particle diameters, respectively, and ΔE and ΔE_0 are the respective amplitudes of the voltage pulses.

If the particle is not a sphere but may be approximated as an ellipsoid of revolution, then its volume, V_e, may be readily determined in terms of the resistively equivalent diameter of equation 2 and a form factor, f_e, viz.,

$$V_e = \pi d^3/4f_e \quad (3)$$

The form factor, f_e, is given by

$$f_e = \frac{1}{1 - n_\perp} + \left(\frac{1}{1 - n_\parallel} - \frac{1}{1 - n_\perp}\right)\cos^2\beta \quad (4)$$

where $n_\perp$ and $n_\parallel$ are extensively tabulated shape-dependent demagnetization factors for a field applied perpendicular and parallel, respectively, to the axis of revolution, and β is the angle between the axis of revolution and the field (27, 32).

The diameter, D, of a pore is given by the implicit equation

$$D \doteq \left\{\frac{4\rho E d_0^3}{\pi R \Delta E(1 + \alpha)[1 - 0.8(d_0/D)^3]}\right\}^{1/4} \quad (5)$$

where R is the resistance of the pore and ρ is the resistivity of the fluid. This equation is derived by combining equation 1 with the resistive length of the pore, namely, with

$$L = \pi R D^2/4\rho \quad (6)$$

If the concentration, C, of particles in a fluid is taken as equal to the time-averaged concentration of particles flowing through the pore, then

$$C = \frac{16\eta\gamma R(J_+ + J_-)}{P\rho D^2} \quad (7)$$

where η is the viscosity of the fluid, γ is the ratio of the effective hydrodynamic length of the pore (the geometric length plus 0.6 D) to the effective resistive length, P is the pressure difference across the pore, and J_+ and J_- are the flow rates of the particles for positive and negative electrical polarities, respectively, of a given field magnitude on the positive-pressure side of the pore.

The concentration of particles in a fluid may also be determined by count comparison with a known number of uniform-sized latex spheres mixed with the particles, viz.,

$$C = \frac{N_s}{V_0}\left(\frac{J_+ + J_-}{J_{s+} + J_{s-}}\right) \quad (8)$$

where N_s is the number of latex spheres added to the volume, V_0, of the suspension of particles, and J_{s+} and J_{s-} are the flow rates of the latex spheres for positive and negative electrical polarities, respectively, on the positive-pressure side of the pore.

ACKNOWLEDGMENTS

We thank C. P. Bean for many valuable discussions and essential ideas, including the initial concept of the analyzer; W. A. Healy for skillful technical help; S. Ludke and G. Jernakoff for expertly designing the needed instrumentation; J. S. Wolff and S. A. Mayyasi for helpful discussions; G. Schidlovsky, T. Liszacak, J. Lelek, and R. R. Russell for electron microscopy measurements; E. O'Brien, C. Higdon, and M. V. Doyle for valuable laboratory assistance; and T. E. O'Connor and J. J. McCormick for linking our laboratories.

This work was supported by contracts NO1-CP-3-3231 and NO1-CP-3-3234 within the Virus Cancer Program of the National Cancer Institute.

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