APPLICATION OF LASER BEAT FREQUENCY SPECTROSCOPY TO THE DETECTION AND CHARACTERIZATION OF AN ONCOGENIC RNA VIRUS

I. Salmeen, D. Gill and L. Rimai
Ford Motor Company, Scientific Research Staff, Dearborn, Michigan
J. J. McCormick, V. M. Maher, W. J. Arnold and M. E. Hight
Detroit Institute of Cancer Research Division, Michigan Cancer Foundation, Detroit, Michigan

Received May 1, 1972

Summary: We have investigated the applicability of laser beat frequency spectroscopy to the detection and characterization of RNA oncogenic viruses. Spectra from suspensions of Soule mouse leukemia virus, both purified and in cell culture harvest fluids, were studied at concentrations in the range of 10^7 to 10^9 particles/cm^3. The spectra were characteristic of the virus and yielded a diffusion constant of $(.34 \pm .02) \times 10^7$ cm^2 sec^{-1} in water at 24°C, from which a hydrodynamic diameter of 136 nm was calculated. By comparing scattered integrated intensities from these preparations with bacteriophage lambda suspensions of known titer, it was determined that tumor virus particles can be readily detected at concentrations of the order of 10^8/cm^3.

Introduction: Laser beat frequency spectroscopy is a technique for measuring the spectral broadening of light scattered from a solution of particles or macromolecules.[1,2] The diffusional motion of the suspended particles causes spectral broadening of the scattered light which, for a monodisperse suspension of rigid particles, corresponds to a Lorentzian frequency distribution with a halfwidth DK^2 centered at the incident frequency. D is an appropriately averaged effective diffusion constant and K is the change in light wave vector caused by the scattering. K is proportional to $\sin(\theta/2)$, where θ is the scattering angle, and D is independent of K for spherical particles.

Biological applications of this technique have been devoted primarily to precision measurements of diffusion constants of proteins,[1,2,3,4] nucleic acids,[4] bacteriophage,[4,5] and tobacco mosaic virus.[4,6] For maximum precision all of this work has been done using high concentrations (10^{12} - 10^{13} particles/cm^3 for the bacteriophages). We describe measurements of an RNA tumor virus at concentrations of 10^7 to 10^{10} particles/cm^3 which yield diffusion constants sufficiently accurate for virus characterization. In

addition, this technique can be used to indicate the presence of such particles and as a means for estimating particle concentrations.

Materials and Methods: The spectral distribution is obtained by frequency analysis of the output current from the photomultiplier tube used to detect the scattered light.[1] Because the photocathode is a square-law detector, the photocurrent is the result of mixing of all incident frequencies. If the only light incident on the photocathode is that scattered from a single type of particles, then the spectrum of the photocurrent will be a Lorentzian distribution of width $2DK^2$ centered at zero frequency (homodyne detection). If, in addition to light scattered from the particles of interest, the photocathode detects light scattered from other much larger and slower moving particles or from cuvette walls, one may, under suitable conditions,[2] observe a Lorentzian distribution of width DK^2 (heterodyne detection). The details of the light scattering spectrometer used in these measurements have been described previously.[2]

In the present measurements, the output of the frequency analyzer was squared and either read point by point, or successive rapid scans were accumulated and read out from the memory of a multichannel analyser. Total sample volumes used were of the order of 0.5 cm^3. Minor modifications would allow reduction to 0.1 cm^3. The cuvettes were washed exhaustively with distilled water passed through a 0.3 micron millipore filter and aseptic techniques were used to transfer virus solutions to the cuvettes. Complete angular dependent measurements on a given sample required about three hours.

Soule murine leukemia virus MuLV(S), was produced in MCF-6 tissue culture cells grown and harvested as previously described.[7] MuLV(S) was purified from cleared medium centrifuged to an interface between 20% and 56% sucrose in a step gradient prepared in standard buffer (0.1 M tris, 0.1 M $NaC\ell$, 0.01 M $MgC\ell_2$, pH 7.4). Interface material was transferred to a continuous sucrose gradient (20 to 56%) and centrifuged to isopycnic equilibrium. The virus-containing band was diluted with standard buffer to

a concentration 25 × that of the original material (this preparation is designated sample a). It contained 20.7% sucrose as measured by index of refraction. A second portion of supernatant from the same culture flask was cleared of cells but not further purified (sample b). Cleared, supernatant from a later passage of MCF-6 was sample c. Unused culture medium (sample d) was also examined as a control. Supernatant from a virus-free mouse cell culture 867B (courtesy of Dr. H. D. Soule), was also studied (sample e). All samples were filtered through a 0.45 micron millipore HA filter, frozen and stored on dry ice.

Bacteriophage lambda was prepared by infecting Escherichia coli C600 grown in tryptone broth to a titer of ca. 3×10^8 cells/cm^3. An m.o.i. of 3 was used to infect the host cells and the culture grown with aeration until cell lysis occurred. Cellular debris was removed by centrifugation and the lysate titered by plaque count using soft agar overlay techniques.[8]

Results and Discussion: To establish a semiquantitative concentration standard we measured the spectra from a solution of bacteriophage lambda with 10^{10} particles/cm^3. At each scattering angle the spectrum could be fitted to a single Lorentzian curve with halfwidth proportional to $\sin^2(\theta/2)$ (Fig. 1, trace λ). The corresponding diffusion constant (at 24°C) is listed in Table I and agrees well with the value previously reported for a high

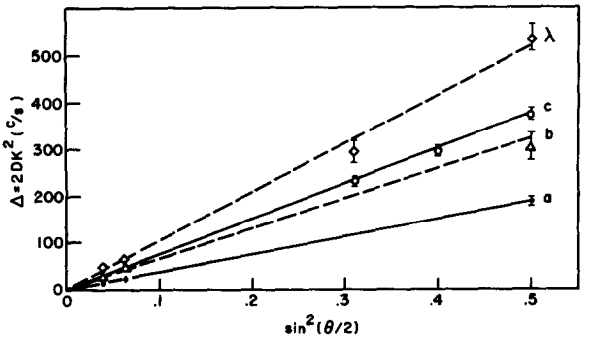

Fig. 1. Angular dependence of linewidths from MuLV(S) samples a, b, and c (described in text) and from bacteriophage lambda. The linewidths shown in b have been multipled by two to correct, as explained in the text, for the heterodyne effect.

TABLE I

Diffusion constants at 24°C. Sample designations described in text.

SAMPLE	D ($\times 10^7$ cm sec^{-1})	HYDRODYNAMIC DIAMETER (nm)
λ	0.46 ± .03	100 ± 10
a	0.17 ± .01	---
a_{corr}	0.34 ± .01	136 ± 5
b	0.30 ± .01	150 ± 30
c	0.34 ± .02	136 ± 5

concentration of lambda.[5] Figure 2 shows examples of spectra obtained from samples a, b, c. Under the same instrumental settings as those used to obtain the data of sample c, the control samples d and e both yielded a

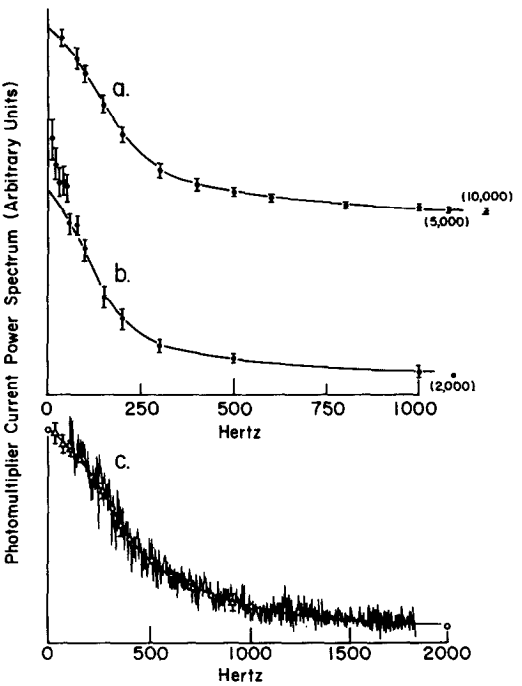

Fig. 2. Representative frequency spectra at 90° scattering angle from the samples a, b, and c. Data in Figs. a and b were taken point by point by averaging the square of the output of the spectrum analyzer as measured by a digital voltmeter. The solid curves are calculated Lorentzian distributions. In Fig. c, the square of the output of the spectrum analyzer was recorded using a signal averager. The noisy solid curve was recorded beginning at 80 Hz with a 10 Hz bandwidth. The solid triangles correspond to data taken at 3 Hz bandwidth beginning at 15 Hz, and normalized to the data beginning at 80 Hz. This low frequency data clearly shows the absence of a low frequency heterodyne signal. The open circles are points on a calculated Lorentzian distribution.

spectrum that was constant above 20 Hz at all angles, indicating the absence of particles with diffusion constants comparable to that of the virus. At each angle, the data for samples a and c could be well represented by single Lorentzian distributions with halfwidths linear in $\sin^2(\theta/2)$ as shown in Fig. 1. The values of D calculated from these data are presented in Table I.

Using the Stokes-Einstein equation[9] for spheres, one can correct for the difference between the 20.7% sucrose solution of sample a and the tissue culture medium of samples b and c (line a_{corr} in Table I). This corrected diffusion constant is in agreement with that derived directly for sample c.

The data from sample b (Fig. 2b) at all angles show pronounced increase in the scattering intensity at frequencies below ca. 80 Hz, in contrast to the results from purified virus (sample a) and another harvest (sample c). If the spectrum above 80 Hz from sample b is fitted to a Lorentzian curve, we find a linewidth about one half of that observed at the corresponding angles for sample c. (In Fig. 1, the widths corresponding to this sample b have been doubled.) This difference in linewidth between samples b and c can be explained if the spectrum of b is interpreted as a heterodyne signal. Two observations suggest such an interpretation. First the large increase in intensity below 80 Hz shows the presence of relatively strong scattering from some source other than the virus particles. Second, the absolute intensity of scattering from sample b above 80 Hz (Fig. 2b) is much less than that from sample c (Fig. 2c). The scattering intensity from c is comparable to that from the purified virus (sample a) which, on the basis of the preparative procedure, is expected to be ca. 25 times more concentrated than b. Since the intensity of a heterodyne spectrum varies linearly as the concentration[2] whereas a homodyne spectrum varies as the square of the concentration, the heterodyne signal would dominate at low concentration.

The effective hydrodynamic diameter in solution can be calculated from the diffusion constants.[9] For MuLV(S) this diameter is 136 nm. MuLV(S)

has shown two size ranges when measured by electron microscopy.[10] Sectioned material exhibits particles ranging in diameter from 80 to 120 nm, averaging 105 nm. Negatively stained whole mounts of virus have shown a range of mean diameters from 102 to 196 nm, averaging 166 nm. The disparity between the two series is due probably to artifacts inherent in the methods of preparation. Sectioned material is usually compressed during cutting and shrinkage owing to dehydration is common to both preparations. Intact but internally unsupported spheres would be expected to flatten and increase in apparent diameter during drying for negative staining. Consequently, the intermediate size calculated in the present study appears to be a reasonable approximation to MuLV(S) diameter.

The presence of spectral broadening with the expected linewidth is a necessary (although not sufficient) condition to indicate the presence of a virus. In addition, the integrated intensity of the scattering from a homodyne spectrum, when properly calibrated, can be used to estimate virus concentration. For our data on sample a, we estimate the concentration (within a factor of 3) at 2×10^8 particles/cm^3 by comparing with the integrated intensity of scattering from bacteriophage lambda of known concentration. (We know the intensity of the broadened homodyne spectrum varies as the square of the concentration and the elastic scattering cross section as the sixth power of the diameter.) Therefore for the parent harvest (sample b), by the heterodyne effect, a concentration of ca. 10^7 particles/cm^3 is calculated whereas for harvest c the concentration is of the order of 10^9 particles/cm^3. These results support the contention that quasielastic scattering, because of its simplicity and relative speed, is a useful tool for detecting and partially characterizing viruses even in cell culture harvests.

REFERENCES

1. S. B. Dubin, J. H. Lunacek, and G. B. Benedek, Proc. Nat. Acad. Sci. (U.S.A.) 57, 1164 (1967).
2. L. Rimai, J. T. Hickmott, T. Cole, and E. B. Carew, Biophysical J. 10, 20 (1970).

3. S. B. Dubin, N. A. Clark, and G. B. Benedek, J. Chem. Phys. 54, 5158 (1971).
4. S. B. Dubin, Ph.D. Thesis, Mass. Inst. of Tech. (1971).
5. S. B. Dubin, G. B. Benedek, F. C. Bancroft, and D. Fraifelder, J. Mol. Biol. 54, (1971).
6. H. Z. Cummins, F. D. Carlson, T. J. Herbert, and G. Woods, Biophysical J. 9, 518 (1969).
7. H. D. Soule and W. J. Arnold, J. Nat. Cancer Inst. 45, 253 (1970).
8. M. H. Adams, Bacteriophages, Interscience, Inc., N. Y. (1959).
9. C. Tanford, Phys. Chem. of Macromolecules, John Wiley & Sons, Inc., N. Y. (1961).
10. W. J. Arnold, To be published.