Molecular Weight Determination by Fluctuation Spectroscopy with Quasi-Elastic Light Scattering

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ABSTRACT: The contribution of number fluctuation in the correlation function and the spectrum was considered for the quasi-elastic light scattering from a finite number of giant molecules in solution. Based on the theoretical results, a new method is proposed for the simultaneous determinations of both molecular weight and diffusion coefficient by measurement of the probability distribution of the photocurrent fluctuation. An application of the method to bacteriophage T4 gave results in good agreement with those of other measurements.

For the determination of molecular weights of giant particles, a simple but absolute method was proposed by Feher and Weissman.[1] They made use of the fact that the number, N, of solute molecules in a small volume, V, in solution fluctuates spontaneously around an average value, $\langle N \rangle$, and that the fractional magnitude of the mean-square fluctuation gives $\langle N \rangle$ as $\langle \delta N^2 \rangle / \langle N \rangle^2 = \langle N \rangle^{-1}$. Thus, from independent measurements of the fractional fluctuation by fluctuation spectroscopy and the concentration (weight per unit volume) of solute molecules, c, the molecular weight is obtained as

$$M = cVN_A/\langle N \rangle \tag{1}$$

where N_A is Avogadro's number. For a given concentration, the fractional fluctuation increases with molecular weight because the number of molecules N becomes smaller. Therefore, this method is more suitable for samples of larger molecular weights. For polydisperse solutions the above formula gives a weight-average value.

To monitor the number of molecules at time t, $N(t)$, with high sensitivity, Weissman et al.[2] labeled the solute molecules (DNA) with a dye (ethidium bromide) and measured the fluorescence intensity. In practice, the photocurrent fluctuation contains several noise components such as the laser output fluctuation, shot noise, dust particles floating in front of the beam, mechanical vibrations, etc. To distinguish the number fluctuation from these unwanted noises, Weissman et al.[2] used a rotating cell and observed only the fluctuation having correlation times longer than a few seconds. The rotating cell technique also allowed data acquistion from statistically independent volumes in a reasonable measuring time. This method is useful for giant DNA molecules for the reasons that extensive purification cannot be applied to such fragile molecules and that many fluorescent dyes are easily bound to DNA by intercalation.

In the present work, we attempted to use quasi-elastic light scattering to monitor the number fluctuation. The advantage of this method is twofold. First, a wider application is expected since labeling with fluorescent substance is unnecessary. Second, there is a possibility to determine the diffusion coefficient, D, as well as the molecular weight. The mean-square photocurrent fluctuation contains a term for the self-beat fluctuation of quasi-elastic light scattering, which is proportional to $\langle N \rangle^2$. In contrast, the term for the spontaneous number fluctuation is proportional to $\langle N \rangle$. Thus, D may be determined from the concentration dependence of the photocurrent fluctuation unless rotational fluctuations and form-factor fluctuations are significant.

It soon became clear, however, that if we use the rotating cell technique even a very slight misalignment of the cell introduces a severe sinusoidal modulation of the correlation function. This difficulty was avoided by using a fixed cell and an electronic filter to reduce the unwanted noises. The fractional photocurrent fluctuation was determined in this case by measuring the probability distribution. It took a longer time for the measurements than with the rotating cell technique, but this is not a serious problem. In this paper we present the principle of the method, the experimental apparatus, and the results of its application to *E. coli* phage T4.

Theory

The output photocurrent, $J(t)$, of a phototube is proportional to the intensity, $I_s(t)$, of light it detects:

$$J(t) = eaI_s(t) \tag{2}$$

Here e is the electronic charge and a is a suitably defined efficiency. The photocurrent fluctuates in proportion to the fluctuation of light intensity. The correlation function of the photocurrent is then given by[3]

$$R_J(t) = e\langle J \rangle \delta(t) + e^2a^2\langle I_s(t)I_s(0) \rangle \tag{3}$$

where the brackets indicate the statistical average. The first term on the right-hand side is the shot noise, which arises because the current is a flow of the corpuscular electronic charge e.[3]

If the number of particles in the scattering volume is finite, the correlation function of the scattered light includes the number fluctuation term.[4] The correlation function $\langle I_s(t)I_s(0) \rangle$ is then given by

$$\langle I_s(t)I_s(0) \rangle = \langle N \rangle^2\beta^2 + \langle N \rangle^2\beta^2 \exp(-2Dk_s^2|t|) + \beta^2\langle \delta N(t)\delta N(0) \rangle \tag{4}$$

where k_s is the scattering vector, β is a suitably chosen scattering efficiency, and D is the diffusion constant of solute molecules. This equation is derived by assuming that we are observing a single coherence area of the illuminated region. If we are observing L areas of coherence, the second term on the right-hand side of eq 4 is reduced[5] by a factor $1/L$. The frequency spectrum of the photocurrent is obtained by the Fourier transformation of the correlation function. The result reads

$$\begin{aligned} S_J(\omega) &= \frac{1}{2\pi}\int_{-\infty}^{\infty} R_J(|t|)\exp(i\omega t)\,dt \\ &= \langle N \rangle \frac{e^2a\beta}{2\pi} + \langle N \rangle^2 e^2a^2\beta^2\delta(\omega) + \\ &\qquad \langle N \rangle^2 \frac{e^2a^2\beta^2}{\pi L}\,\frac{2Dk_s^2}{\omega^2 + (2Dk_s^2)^2} + e^2a^2\beta^2 S_N(\omega) \end{aligned} \tag{5}$$

Here $S_N(\omega)$ denotes the Fourier transform of $\langle \delta N(t)\delta N(0) \rangle$.

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The functional forms of $\langle \delta N(t)\delta N(0)\rangle$ were calculated for some cases.[6,7] In this work, it suffices to note that the characteristic relaxation time of this function is of the order of $d^2/2D$, where d is the diameter of the scattering volume. Naturally, d is much longer than the wavelength λ of the laser light; $d \gg \lambda$. It follows, therefore, $Dk_s^2 \ll D/d^2$. In this case, $S_N(\omega)$ is finite only at low frequencies. We introduce a low-pass filter and cut off the fluctuations having frequencies larger than ω_c. Here ω_c is chosen so that $D/d^2 \ll \omega_c \ll Dk_s^2$. Then the high-frequency fluctuations of the first and the third terms in eq 5 are smoothed out, whereas the second and the last terms remain unaffected. Approximating the filter function by a Lorentzian, we have

$$S_J'(\omega) = \langle N\rangle \frac{e^2a\beta}{2\pi} \frac{\omega_c^2}{\omega^2 + \omega_c^2} + \langle N\rangle^2 e^2a^2\beta^2\delta(\omega) + \langle N\rangle^2 \frac{e^2a^2\beta^2}{2\pi LDk_s^2} \frac{\omega_c^2}{\omega^2 + \omega_c^2} + e^2a^2\beta^2 S_N(\omega) \quad (6)$$

By Fourier inversion we have

$$R_J'(t) = \langle N\rangle \frac{e^2a\beta\omega_c}{2} \exp(-\omega_c|t|) + \langle N\rangle^2 e^2a^2\beta^2 + \langle N\rangle^2 \frac{e^2a^2\beta^2\omega_c}{2LDk_s^2} \exp(-\omega_c|t|) + e^2a^2\beta^2\langle \delta N(t)\delta N(0)\rangle \quad (7)$$

The second term on the right-hand side is $\langle J\rangle^2$. The sum of the other three terms expresses the fluctuation of the photocurrent. Setting $t = 0$, we get $\langle \delta J^2\rangle$. The first term is the contribution of the shot noise, and the ratio of the first to the second term is $e\omega_c/\langle J\rangle$. When we chose ω_c as 10 Hz, the magnitude of $\langle J\rangle$ was found to be about 10^{-9} A. In this condition $e\omega_c/\langle J\rangle \sim 10^{-8}$ and the effect of the shot noise can be neglected. $\langle \delta N(0)\delta N(0)\rangle = \langle N\rangle$. Then we get

$$\langle J\rangle^2 = \langle N\rangle^2 e^2a^2\beta^2 \quad (8a)$$

$$\langle \delta J^2\rangle = \langle N\rangle e^2a^2\beta^2 + \langle N\rangle^2 e^2a^2\beta^2\omega_c/2LDk_s^2 \quad (8b)$$

The probability P_N that N noninteracting molecules will be found in the illuminated region is given by the Poisson distribution. In fact, Schaefer and Pusey[8] observed non-Gaussian statistics of the scattered light due to the number fluctuation for $\langle N\rangle = 3.37$. However, if the mean number $\langle N\rangle$ is sufficiently large and the fluctuations concerned are not far from $\langle N\rangle$, P_N can be approximated by a Gaussian distribution. The error introduced by this approximation for $\langle N\rangle = 20$ (the smallest number used for the observation in this study) was estimated as less than 1.5%. Then the probability distribution of the photocurrent may have a Gaussian form

$$P(J) = (1/\pi)(\langle \delta J^2\rangle)^{-1/2} \exp[-(J - \langle J\rangle)^2/\langle \delta J^2\rangle] \quad (9)$$

We can determine the relative values of $\langle J\rangle^2$ and $\langle \delta J^2\rangle$ by fitting experimental data of $P(J)$ to eq 9. It should be pointed out that when $\langle N\rangle$ is not too large, say less than 1000, the second term in eq 8b is usually small compared with the first because of the factor of $(\omega_c/LDk_s^2) \ll 1$.

From eq 1 and 8, we finally obtain

$$\frac{\langle \delta J^2\rangle}{\langle J\rangle^2} c = \frac{M}{VN_A} + \frac{\omega_c}{2LDk_s^2} c \quad (10)$$

This equation shows that the plot of the quantity on the left-hand side vs. c gives a straight line. The solute molecular weight M may be estimated from the intercept with the vertical axis if the scattering volume V is known from independent measurements. Similarly, the slope of the plot gives the diffusion coefficient D if $\omega_c/2Lk_s^2$ is known.

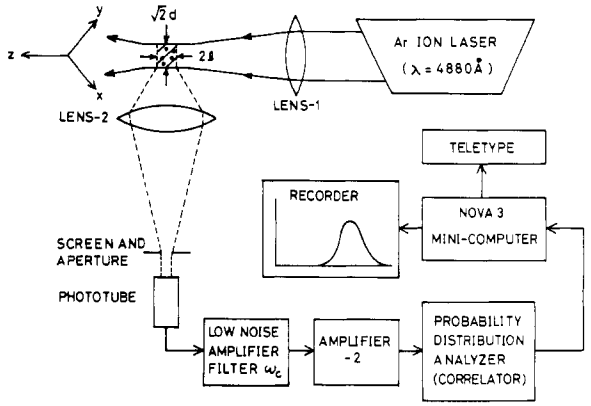

Figure 1. Block diagram of the experimental arrangement for the determination of the probability distribution of the photocurrent fluctuation for the quasi-elastic light scattering.

If the molecule is very anisotropic in shape, the rotational relaxation affects the current correlation function, and we need to take into consideration additional relaxation functions. In the case of tobacco mosaic virus, for example, the dominant correction term for the correlation function is proportional to $\exp(-6D_R t)$, with the rotational diffusion coefficient $D_R = 320 \sim 420$ s^{-1}.[9] This is a function decaying much faster than the translational number fluctuation. By electric filtering, the amplitude of this rotational fluctuation can be reduced by a factor of $\omega_c/6D_R \simeq 5 \times 10^{-3}$, which can be neglected. However, for very accurate studies of the molecular weights of nonspherical molecules, we need a further extension of the present study. The same is true for flexible molecules.

Experimental Section

Optics. A block diagram of the experimental apparatus is shown in Figure 1. The light source is an Ar ion laser, Model GLG3200, Nippon Electric Co. Its output power was controlled constant at 25 mW for the light of 4880 Å. The laser beam was focused at the center of the sample cell with a lens with a focal length of 50 mm (lens 1). A conventional quartz cell (10 × 10) for UV absorption measurements was used as the sample cell. The light scattered by a sample solution at right angles to the incident beam was collected with another lens with a focal length of 15 mm (lens 2), and a magnified image of the laser beam was formed on a screen. The magnification was 45. The length $2l$ of the scattering volume was determined by the width, 0.4 mm, of a small aperture on the screen.

Electronics. The scattered light that passed through the aperture was detected with a photomultiplier tube, Type R268, Hamamatsu Television. A low-noise amplifier, Model 113, Princeton Applied Research, was used to amplify the output signal from the phototube and to filter the high-frequency fluctuation. The low-pass cutoff frequency, ω_c, was determined by the low-pass filter of the amplifier. The output signal was further amplified with an operational amplifier, Type 741 (amplifier 2). The output of the second amplifier was fed into a homemade correlator, which is a modification of a correlator reported previously.[10] The correlator is capable of calculating the probability distribution, the probability integral, and the auto- and cross-correlations of both the digital and analog signals. In the present experiment, the correlator was used as a calculator of the probability distribution of analog signals. The data accumulated in the correlator was transferred to a Nova/3 minicomputer, Japan Data General, for the on-line control and for plotting the experimental and calculated results.

Materials. Uniform polystyrene latex particles were obtained from Dow Chemical Co. The catalog diameter is 0.091 μm, and the density is 1.05 g/mL. Dilute latex suspensions for the light scattering measurements were prepared from a stock suspension (10% solids) as follows.[11] A small amount of latex was diluted with freshly prepared double-distilled water in a glass centrifuge

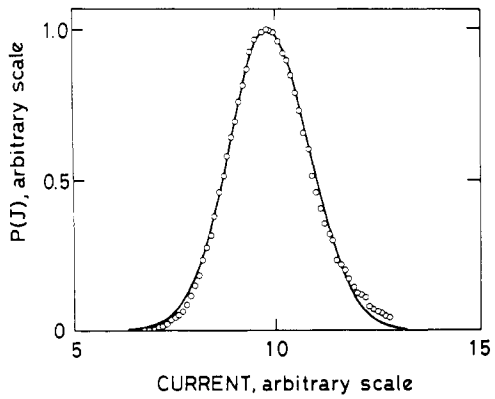

Figure 2. Probability distribution of the photocurrent fluctuation of laser light scattered by polystyrene latex spheres with a diameter of 0.091 μm. The weight concentration was 1.29×10^{-6} g/mL. The amplification factor used was 200 for the low-noise amplifier and 10 for amplifier 2. The open circles represent the observed data, and the full curve is the best-fit Gaussian distribution determined by the method of least squares. The ratio $\langle \delta J^2 \rangle / \langle J \rangle^2$ was evaluated as 0.0113.

bottle of 40-mL capacity to reduce the concentration to about 1%. The dilute latex was then centrifuged at about 350*g* for 20 min to remove larger aggregates. The polymer concentration was determined by accurately weighing out about 10 mL of this suspension and evaporating it to dryness in a partial vacuum at 80 °C and then weighing the dried solids. A series of dilutions was made by weight from the remainder of the centrifuged latex by adding double-distilled water.

T4 phages were prepared from lysates of *E. coli* B^E infected with T4 D^+. The strains were donated by M. Yanagida (Kyoto University). The phage was purified by four cycles of high- and low-speed centrifugation and two cycles of isopycnic centrifugation in CsCl containing 1 mM $MgSO_4$. Phages were then extensively dialyzed against 20 mM KH_2PO_4, 50 mM Na_2HPO_4, 70 mM NaCl, and 1 mM $MgSO_4$. The final dialysate was always saved for use as spectrophotometric blanks and as solvent blanks for light scattering measurements. Both solvent and phage solutions were filtered through 0.22-μm Millipore filters which had been prewashed in 0.1% bovine serum albumin to avoid loss of phage by adsorption.[12] The phage concentration of the filtrate was determined spectrophotometrically with $\epsilon = 0.0314$ cm²/g at 260 nm.[13] The sample solutions for the light scattering measurements were prepared by diluting by weight the filtered phage stock solutions in the cleaned sample cell.

Results and Discussion

Calibration of V and ω_c/Lk_s^2 with Polystyrene Latex Spheres. Polystyrene latex spheres of known diameter, from Dow Chemical Co., were usually used in calibrating the light scattering apparatus.[1] The diameter of the sample we used was 0.091 μm. The diffusion coefficient at 20 °C in water was calculated by Stokes' law to be 4.75×10^{-8} cm²/s. The molecular weight was calculated to be 2.50×10^8, using a value of 1.05 for the density. The probability distribution of the photocurrent due to the fluctuation in the quasi-elastic light scattering was measured at various concentrations, and the effective values of the scattering volume V and the scattering factor ω_c/Lk_s^2 were empirically determined by fitting the obtained results to eq 10.

The measurements were made at five concentrations ranging from 6.97×10^{-7} to 5.21×10^{-6} g/mL. A typical result of the probability distribution is shown in Figure 2 for the concentration $c = 1.29 \times 10^{-6}$ g/mL. The gains of the two amplifiers were adjusted so that an appropriate voltage was fed to the correlator whose full scale was 5 V. The sampling time of the correlator, during which the photocurrent is integrated as a sample point, was 100 μs. This is much shorter than the relaxation time of the fluctuation to be considered in the present study. The samplings were repeated for 20 min to obtain a probability distribution result. A similar sampling time and measuring time were used throughout the present study. The obtained distribution was fitted to a Gaussian distribution, eq 9, by the method of least squares to evaluate the values for $\langle J \rangle$ and $\langle \delta J^2 \rangle$. A small contribution (about 3% at $c = 1.29 \times 10^{-6}$ g/mL) from the solvent scattering was subtracted from $\langle J \rangle$ so determined, and the ratio $\langle \delta J^2 \rangle / \langle J \rangle^2$ was finally evaluated. Similar measurements were repeated for each sample until 5–20 good data were accumulated, and their average was plotted in Figure 3 against concentration c.

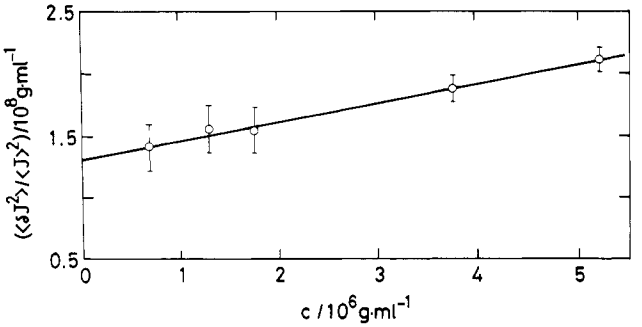

Figure 3. Plots of $(\langle \delta J^2 \rangle / \langle J \rangle^2)c$ vs. c for polystyrene spheres 0.091 μm in diameter, where $\langle \delta J^2 \rangle$ is the mean-square fluctuation of photocurrent and $\langle J \rangle$ is the mean dc photocurrent. The latter was corrected for the small contribution of the solvent scattering. The straight line was drawn by the method of least squares, yielding 1.31×10^{-8} g/mL and 1.50×10^{-3} for the ordinate intercept and the slope, respectively.

In good agreement with the theoretical relation, eq 10, the plotted points fall on a straight line, and the ordinate intercept and the slope of the best fit were obtained as $(1.31 \pm 0.10) \times 10^{-8}$ g/mL and $(1.50 \pm 0.12) \times 10^{-3}$, respectively. By combining these results with the molecular weight and the diffusion coefficient of polystyrene spheres calculated above, we obtain

$$V = (3.2 \pm 0.3) \times 10^{-7} \text{ mL}$$
$$\omega_c/2Lk_s^2 = (7.1 \pm 0.6) \times 10^{-11} \text{ cm}^2/\text{s} \quad (11)$$

In Figure 2, some deviations from the Gaussian distribution are observed at tails of the distribution. These deviations might be improved if we use the Poisson distribution. However, the experimental error by the approximation can be neglected in the experimental conditions of the present study, as discussed in the theoretical section.

Molecular Weight Determination of T4 Phage. As a demonstration of the feasibility of the present method, the molecular weight and the diffusion coefficient of *E. coli* phage T4 were determined by the present method and the results were compared with literature values. The measurements were made at four concentrations in the range 1.83×10^{-7} to 1.84×10^{-6} g/mL. Figure 4 shows a typical current distribution for $c = 7.6 \times 10^{-7}$ g/mL. The experimental conditions were the same as for the measurements of polystyrene spheres. Some deviation of the data points from the Gaussian distribution in the tail region of the larger currents may indicate effects from contaminating dust particles. These data points were omitted in determining the Gaussian distribution (full curve) by the method of least squares. The ratio $\langle \delta J^2 \rangle / \langle J \rangle^2$ was estimated after a small correction (6.7% at $c = 7.6 \times 10^{-7}$ g/mL) for the solvent scattering to $\langle J \rangle$ was made.

The results thus obtained are plotted in Figure 5 against c. The plotted points scatter more than in polystyrene

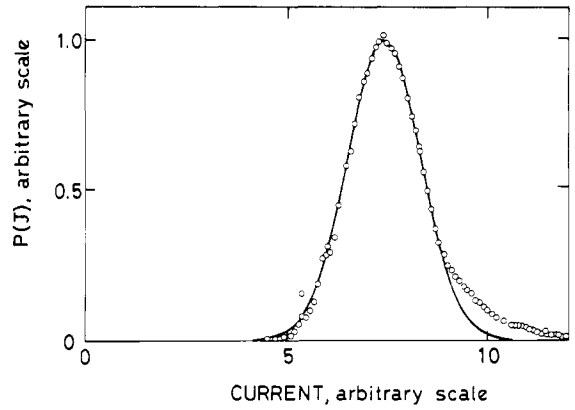

Figure 4. Probability distribution of photocurrent distribution of laser light scattered by *E. coli* phage T4. The weight concentration was 7.6×10^{-7} g/mL. The amplification factor was 500 for the low-noise amplifier and 10 for amplifier 2. The ratio $\langle \delta J^2 \rangle / \langle J \rangle^2$ was evaluated as 0.0169. For details see the legend to Figure 2.

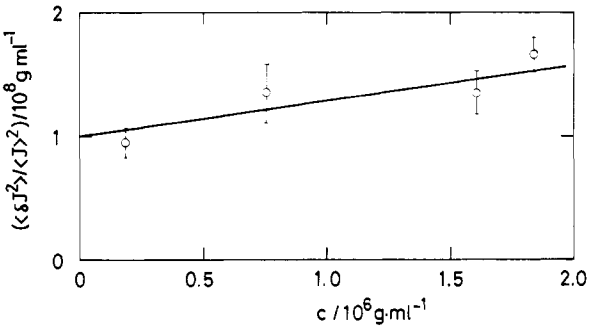

Figure 5. Plots of $(\langle \delta J^2 \rangle / \langle J \rangle^2)c$ vs. c for *E. coli* phage T4. The ordinate intercept and the slope of the best-fit straight line are evaluated as 1.0×10^{-8} g/mL and 2.8×10^{-3}, respectively. For details see the legend to Figure 3.

latex measurements, for reasons to be discussed later. The ordinate intercept and the slope were determined by the method of least squares as $(1.0 \pm 0.1) \times 10^{-8}$ g/mL and $(2.8 \pm 0.3) \times 10^{-3}$, respectively. Using the calibration constants of eq 11, we finally obtain the results

$$M = (1.9 \pm 0.2) \times 10^8$$

$$D = (2.5 \pm 0.3) \times 10^{-8} \text{ cm}^2/\text{s}$$

These results are in satisfactory agreement with the data $M = (1.925 \pm 0.066) \times 10^8$ and $D = (2.95 \pm 0.03) \times 10^{-8}$ cm^2/s, reported by Dubin et al.[12] They measured D using optical mixing spectroscopy and determined M by calculation with D and the sedimentation coefficient.

The experimental errors of about 10% for both the M and D determinations are still large and need to be improved. The contamination of dust particles in T4 phage solutions, as mentioned above, might be due to imperfect exclusion with Millipore filters, but this effect may be less severe for samples of larger sizes. What is more important is the lack of complete reproducibility of the measurements of $\langle \delta J^2 \rangle$. One possible explanation is that the measuring time of 20 min is not sufficiently long. The time that molecules take to move across the scattering volume may be estimated from $\tau = d^2/2D$. For polystyrene spheres it is about 7 min. During this time interval, the number of molecules in the scattering volume is correlated, and the samplings are not statistically independent. This consideration indicates that we observe only 3 statistically independent volumes in the measuring time. A repetition of 20 such measurements would yield 60 different sampling volumes. For T4 phages this number reduces to 30. This partly explains why the data for T4 phage scatter more. In order to attain higher accuracy, a longer measuring time may be required, but the instability of the apparatus sets a practical limit. More simply, the difficulty may be avoided by giving a small vertical translational motion to the sample cell during a measurement. This modification, which we are now undertaking, may increase the accuracy of the measurement and allow this method to be applied to larger molecules.

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References and Notes

(1) Feher, G.; Weissman, M. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 870.
(2) Weissman, M.; Schindler, H.; Feher, G. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 2776.
(3) See, for example: Chu, B. "Laser Light Scattering"; Academic Press: New York, 1974; p 93.
(4) Schaefer, D. W.; Berne, B. J. *Phys. Rev. Lett.* **1972**, *28*, 475.
(5) Benedek, G. B. *Brandeis Univ. Summer Inst. Theor. Phys., 1966* **1968**, 97.
(6) Madge, D.; Elson, E.; Webb, W. W. *Phys. Rev. Lett.* **1972**, *29*, 705.
(7) Schaefer, D. W. *Science* **1973**, *180*, 1293.
(8) Schaefer, D. W.; Pusey, P. N. *Phys. Rev. Lett.* **1972**, *29*, 843.
(9) Cummins, H. Z. In "Photon Correlation and Light Beating Spectroscopy"; Cummins, H. Z., Pike, E. R., Eds.; Plenum Press: New York and London, 1974; p 285.
(10) Ohbayashi, K. *Jpn. J. Appl. Phys.* **1974**, *13*, 1219.
(11) Heller, W.; Tabibian, R. *J. Phys. Chem.* **1962**, *66*, 2059.
(12) Dubin, S. B.; Benedek, G. B.; Bancroft, F. C.; Freifelder, D. *J. Mol. Biol.* **1970**, *54*, 547.
(13) Bancroft, F. C.; Freifelder, D. *J. Mol. Biol.* **1970**, *54*, 537.