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Intensity Fluctuation Spectroscopy of Laser Light Scattered by Solutions of Spherical Viruses: R17, $Q\beta$, BSV, PM2, and T7. I. Light-Scattering Technique†

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ABSTRACT: The technique of digital autocorrelation of intensity fluctuations in scattered laser light has only recently been applied to biological problems. It affords precise and rapid measurements of the translational diffusion constants of macromolecules; typically, an accuracy of 1% can be achieved in about 1 min. This paper is intended to be sufficiently detailed and to contain sufficient information to enable workers in the field of biophysical chemistry to assess the applicability of the technique to problems of their interest. We discuss the origin of intensity fluctuations in laser light scattered by a macromolecular solution and give a brief review of the theory of photocount autocorrelation. We then describe the apparatus, and the methods for the reduction of experimental data. In addition to discussing the least-squares data analysis, we present a general method by which various spurious effects such as dust in the sample, and real effects, such as macromolecular polydispersity, may be detected and characterized. Finally we consider sources of error in the measurement of D and present typical data for solutions of R17 virus to illustrate the performance of our apparatus.

The new technique of digital autocorrelation of intensity fluctuations in scattered laser light affords precise and rapid measurement of macromolecular diffusion coefficients (Jakeman and Pike, 1969; Foord *et al.*, 1970; Pusey *et al.*, 1972), values for which can typically be obtained with an accuracy of 1% in about 1 min. We have used this technique to

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measure the translational diffusion coefficients of the spherical viruses R17, Qβ, PM2, T7, and BSV (tomato bushy stunt virus). The work will be described in two parts: the present paper concerns the light scattering method and the following paper (Camerini-Otero *et al.*, 1974) contains the bulk of the virus data and a discussion of the physical and biophysical implications of the results.

A physical picture of the origin of intensity fluctuations in light scattered by a solution of macromolecules can be obtained by analogy with the scattering of X-rays by a crystal (*cf.* Clark *et al.*, 1970). Though oversimplified, this picture illustrates many of the important features of the phenomenon. Atoms or molecules in a highly ordered crystalline array scatter a monochromatic beam of X-rays to give a diffraction pattern containing relatively few, but intense, maxima. By contrast, macromolecules in solution are, at any instant, randomly located. This random placement may be regarded as a (Fourier) superposition of many crystalline arrays of varying geometry and orientation, so that the diffraction pattern arising from the scattering of monochromatic light by such a solution consists of a superposition of many randomly placed maxima and minima of varying size and intensity. Moreover, unlike atoms in a crystal, the macromolecules are free to diffuse throughout the solution (Brownian motion). Hence the diffraction pattern of the scattered light will fluctuate with time. Indeed, if the scattered light is sufficiently intense, a fluctuating "speckle" pattern can be observed with the naked eye on a screen set a few inches away from the scatterer. A detector of the scattered light, having a photosensitive area roughly equal to the spatial extent of one diffraction maximum (one coherence area), will therefore perceive a randomly fluctuating light intensity whose evolution in time will reflect the Brownian diffusion rates of the macromolecules. An estimate of the time scale of these fluctuations is the average time taken for a diffraction minimum to replace a maximum at the detection point, and is clearly of the order of the time it takes a macromolecule to diffuse one wavelength of light. This (coherence) time is in the microsecond to millisecond range for most solutions of biological macromolecules.

The experimental observation of intensity fluctuations in light scattering has been made possible by the advent of lasers as intense, monochromatic light sources. We shall not attempt a comprehensive review of the development of the field, but shall restrict ourselves to a few relevant comments. Reference can be made to recent review articles (Benedek, 1969; Cummins and Swinney, 1970; Chu, 1970; Ford *et al.*, 1974; Ford, 1972; Pecora, 1972; Jakeman and Pike, 1974), several of which contain extensive bibliographies. Other recent work of biological interest, not mentioned elsewhere in this paper, are studies of the following macromolecules: bacteriophages T4, T5, T7, and λ (Dubin *et al.*, 1970), RNase (Rimai *et al.*, 1970), lysozyme (Dubin *et al.*, 1971), casein micelles (Lin *et al.*, 1971, Tamm-Horsfall glycoprotein (Oliver *et al.*, 1971), ribosomes (Vournakis and Rich, 1971; Koppel, 1973), and myosin (Herbert and Carlson, 1971).

There are two complementary methods for analyzing the time dependence of detected light of randomly fluctuating intensity. One can measure the frequency spectrum of fluctuations in the detector photocurrent; or one can measure the time autocorrelation function of individually detected photons (or photocounts), and thereby determine the range in time over which the intensity fluctuations are correlated. (The data obtained by these two methods form a Fourier-transform pair and are physically equivalent.) The frequency analysis method, which until fairly recently has been the more widely used, suffers from two major drawbacks. First the method is inherently sensitive to errors due to instrumental nonlinearities, since it is basically an analog procedure which treats the detector photocurrent as if it were continuous. Second, many frequency analyzers are single-channel devices which require long experimental run times to scan the appropriate spectral range. On the other hand, multichannel photocount autocorrelation, a method made practical by recent advances in integrated circuit technology, not only takes advantage of the discrete, or photon, nature of detected light, but also computes many points of the correlation function simultaneously. In practice the photocount correlation method, used in this work, achieves speed and accuracy close to the theoretical maximum.

For either method of analysis, two detection methods may be used in the study of intensity fluctuations: homodyning and heterodyning. In the heterodyne method, the scattered light is mixed at the detector surface with an excess of unscattered laser light, whereas in the homodyne method only the scattered light itself is studied. While heterodyning has advantages in some experimental situations, in this paper we will be mainly concerned with homodyne detection. In much of the earlier literature, where emphasis has been placed on the frequency spectrum of the scattered light rather than its time correlation function, the study of intensity fluctuations has been called "light-beating" or "optical-mixing" spectroscopy. These names have their origin in the fact that intensity fluctuations in a light beam can be regarded as being due to beats between the different frequency components of the electric field. In the heterodyne method beating occurs predominantly between the frequency components of the scattered electric field and the single frequency of the direct laser field. In the homodyne method the scattered electric field beats against itself, hence the term "self-beat."[1]

Finally, we should mention that while we deal mainly with translational diffusion in this paper, this is by no means the only process of biological interest which can be studied by intensity fluctuation spectroscopy. For example, for nonspherical macromolecules of size comparable to the wavelength of light, rotational diffusion (Pecora, 1968; Cummins *et al.*, 1969; Fujime, 1970; Schaefer *et al.*, 1971) and flexing motions of the molecules (Pecora, 1965; Fujime and Ishiwata, 1971) contribute to the time dependence of the scattered light. The motion of self-propelled organisms (see, for example, Nossal *et al.*, 1971; Schaefer, 1973) and macromolecules moving under the influence of electric fields (Ware and Flygare, 1971; Uzgiris and Costaschuk, 1973) can also be studied by intensity fluctuation spectroscopy.

Method of Photocount Autocorrelation

The photocount autocorrelation technique has been discussed in some detail by, among others, Jakeman and Pike (1969), Foord *et al.* (1970), Koppel (1971), and Jakeman and Pike (1974). Here we assemble the important concepts relevent to macromolecular solutions.

Photocount autocorrelation can be understood by referring to Figure 1 (see also Apparatus). The first line of Figure 1

[1] It should be mentioned that it is not necessary to use a truly single-frequency laser source. Intensity fluctuations in light scattering can, in fact, be observed using a laser operating in several longitudinal modes, each one of which can undergo significant phase fluctuation. The optical bandwidth of such a laser can be many orders of magnitude greater than the broadening induced by the scatterer (*cf.* Cummins and Swinney (1970) and Mandel (1969)).

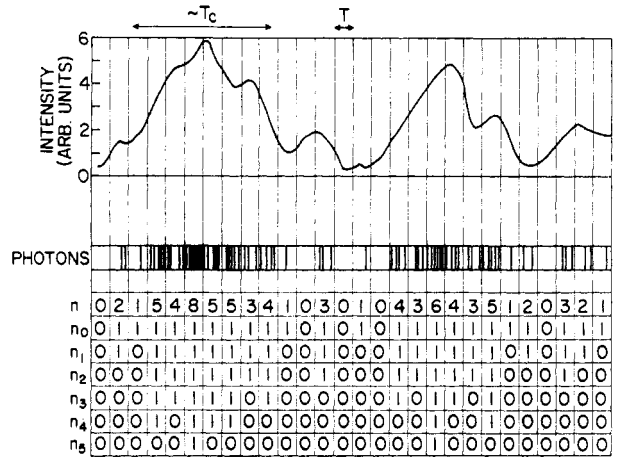

FIGURE 1: Typical trace of fluctuating light intensity, and typical distribution of detected photons (photocounts). The unclipped photon number, n, and clipped photon number, n_k, for clipping levels 0–5, are also shown.

shows a typical trace of light of fluctuating intensity; about three coherence times T_c are spanned in this figure. At any instant, the probability (per unit time) of detecting a photon is directly proportional to the intensity (see, for example, Mandel and Wolf, 1965). The second line shows a typical distribution of detected photons or photocounts, per small time interval of length T. The bunching of the photons due to the fluctuating intensity is evident; when the intensity is high, many photons are detected per interval, and *vice versa*. We define $n(t_i)$ to be the number of photons detected in the sampling interval i, centered at time t_i. The various values of n for the example of Figure 1 are shown in line three. A "full" autocorrelator would construct the sums of products

$$\sum_{i=M}^{N} n(t_i)n(t_{i-m})$$

for a range of delay times $m\mathbf{T}$, where $m = 1, 2, 3, \cdots, M$, and M is the number of channels built into the autocorrelator. N is the number of sampling intervals of length T in the experimental run. In practice, one generally uses "clipped" rather than full correlation, the clipped count $n_k(t_i)$ defined by

$$n_k(t_i) = 1 \quad \text{if } n(t_i) > k$$
$$n_k(t_i) = 0 \quad \text{if } n(t_i) \leq k$$

where the nonnegative integer k is the "clipping level." A single-clipped correlator then measures

$$c_m \equiv \sum_{i=M}^{N} n(t_i)n_k(t_{i-m}) \tag{1}$$

Figure 1 shows the values of n_k for $0 \leq k \leq 5$. The advantage of clipped over full correlation is that it allows the past history of photocounts to be stored as a linear chain of 1's and 0's, as shown in Figure 1, leading to considerable simplification in the circuitry of the correlator, with little loss in the physical information desired. Provided c_m and $N\mathbf{T}/T_c$ are large, any one measured value of c_m will be close to the average value obtained from a large ensemble of experiments

$$\langle c_m \rangle = N\langle n(t_i)n_k(t_{i-m})\rangle \tag{2}$$

(See Data Analysis for a further discussion of this point.) When the number of scatterers is large, the scattered electric field $E(t)$ has a Gaussian amplitude distribution (see, for example, Cummins and Swinney, 1970; also Schaefer and Berne, 1972) and the single-clipped correlation function is given by (Jakeman and Pike, 1969; Koppel, 1971)

$$\langle n(t_i)n_k(t_{i-m})\rangle = \langle n\rangle\langle n_k\rangle\left[1 + \frac{(1+k)}{(1+\langle n\rangle)}\beta|g(m\mathbf{T})|^2\right] \tag{3}$$

where

$$|g(m\mathbf{T})| \equiv \frac{|\langle E^*(t)E(t+m\mathbf{T})\rangle|}{\langle|E(t)|^2\rangle} \tag{4}$$

the normalized electric field autocorrelation function, is the experimental quantity of interest. For light scattered by a solution of identical, noninteracting particles which are spherically symmetric and/or small compared to the wavelength of the incident light

$$|g(m\mathbf{T})| = \exp(-\Gamma m\mathbf{T}) \tag{5}$$

and $\Gamma \equiv 1/T_c$, the decay rate of the electric field (amplitude) fluctuations, is given by (Pecora, 1964; see also Cummins *et al.*, 1969)

$$\Gamma = DK^2 \tag{6}$$

Here D is the translational diffusion coefficient of the macromolecules, and K, the magnitude of the scattering vector, is given by

$$K = (4\pi/\lambda_0)n_0 \sin(\theta/2) \tag{7}$$

where n_0 is the solution refractive index, θ is the scattering angle, and λ_0 is the wavelength *in vacuo* of the incident light.

The factor β (usually $\beta \simeq 1$) in eq 3 takes into account such effects as incomplete spatial coherence of the light over a finite size detector, and detector dark current (Koppel, 1971). It is a complicated function of $\langle n\rangle$, k, $m\mathbf{T}$, $\Gamma\mathbf{T}$, and the geometric arrangement of the optical components. However, when $|g(m\mathbf{T})|$ has the simple exponential form of eq 5, β is independent of m, and can thus be regarded as a constant parameter to be determined experimentally for a given experimental run (Koppel, 1971; see also Jakeman *et al.*, 1971b). Then, combining eq 2, 3, and 5, the experimental quantities c_m are related to the desired decay rate Γ through the equation

$$\langle c_m \rangle = N\langle n\rangle\langle n_k\rangle[1 + \gamma \exp(-2\Gamma m\mathbf{T})] \tag{8}$$

where

$$\gamma = \beta(1+k)/(1+\langle n\rangle) \tag{9}$$

Apparatus

Light-Scattering Photometer. Figure 2 shows schematically the light-scattering photometer. The light source was a Coherent Radiation Laboratories Model 52K krypton ion laser, factory equipped with an intensity-stabilizing feedback loop which holds fluctuations in the emitted intensity within 1%. The electric field of the light is polarized perpendicular to the scattering plane. The yellow-green 5682-Å line, having a maximum power of about 100 mW, was used for most of the work reported in the accompanying paper (Camerini-Otero *et al.*, 1974). After reflection by an adjustable mirror, the laser beam was focussed into the sample by a 20-cm focal length lens. The sample cell, a standard 1 × 1 cm square fluorimeter cell, was mounted on the axis of a cylindrical water bath, about 15 cm in diameter. Using, as we do, a small sample volume, $\lesssim 1$ cm^3, we have found a square sample cell to be markedly superior to a cylindrical cell for minimizing the amount of stray laser light scattered by the walls of the cell.

The temperature of the bath water was controlled to a few

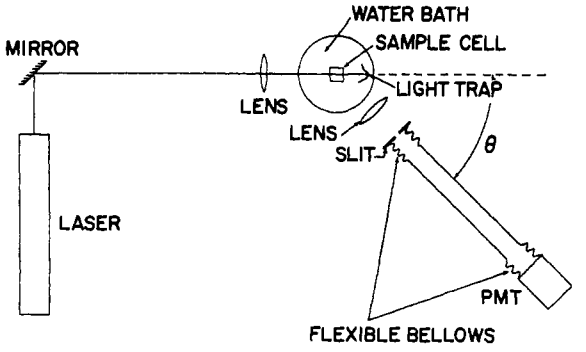

FIGURE 2: The light-scattering photometer.

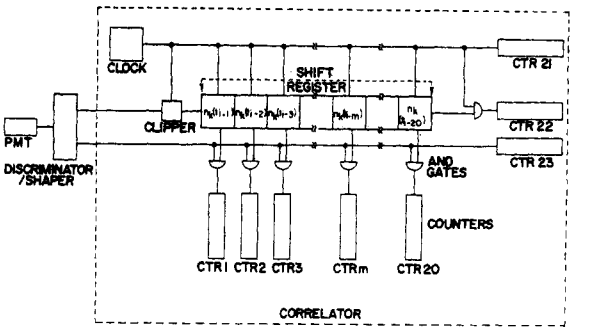

FIGURE 3: Block diagram of the correlator.

hundreths of a degree, and was measured with an NBS-standardized mercury-in-glass thermometer. Absolute temperature uncertainty was about 0.1°. The water was filtered through a 0.45 μ Millipore filter to remove dust which might otherwise interfere with the incident or scattered light, and was changed several times a day. The transmitted laser beam was trapped to minimize stray reflections. A second lens of focal length 5 cm imaged the sample cell onto an adjustable slit of typical width 0.5 mm and height 2 mm, which served to block light other than that scattered from the center of the cell. An ITT FW130 photomultiplier tube, having a circular photocathode 0.35 cm in diameter, was placed about 1 m behind the slit. This arrangement of optical components was such that photomultiplier collected the scattered light over roughly one coherence area. The lens-slit–PMT system was mounted on an optical bench free to rotate about the axis of the water bath, over a scale calibrated in degrees. Thus, the scattering angle was easily variable without loss of system alignment, and was defined to a few tenths of a degree. The whole photometer was mounted on a heavy, rigid table. No special vibration isolation was found to be necessary.

The current output of pulses from the PMT, each resulting from the detection of a single photon,[2] were amplified and shaped (25-nsec width, 5-V amplitude) by standard nuclear physics instrumentation. Experiments were run under normal room illumination, the background count rate being less than 100/sec. This low value was achieved by mounting the slit and PMT at opposite ends of a light-tight assembly of a blackened brass tube and flexible bellows. The counting rate from solvent alone was typically a few hundred per sec.

Description and Operation of the Correlator. Figure 3 shows a block diagram of our 20-channel correlator (*i.e.*, $M = 20$), which is similar to that described by Foord *et al.* (1970). The detailed circuitry of the correlator, which incorporates some 400 Signetics TTL integrated circuits, will be described elsewhere (Koppel, 1973); here we summarize its operation. Time digitization is provided by a crystal clock whose basic frequency, 4.0000 ± 0.0004 MHz, can be digitally divided by 1, 10, or 100 × (1, 2, 3, ····, 100), giving a minimum frequency of 400 Hz. The past history of clipped photocounts is stored in a 20-stage, one-bit-per-stage shift register, SR. Figure 3 shows the state of the correlator during the sampling interval t_i. The number of clipped counts stored in the first stage of SR is $n_k(t_{i-1})$ (= 0 or 1), the number in the second stage $n_k(t_{i-2})$, etc. The real-time multiplication between delayed clipped photocounts and undelayed unclipped photocounts is performed by simple AND gates. The totality of pulses emitted by the discriminator–shaper during sampling interval t_i will be counted by counter m if, and only if, $n_k(t_{i-m})$ = 1. At the end of interval t_i, the contents of SR are shifted one stage to the right, and the process is repeated during interval t_{i+1}. Counter m therefore accumulates the product of the unclipped counts in one interval with the clipped counts m intervals earlier. Each of the 20 correlation counters has 20-bit capacity, corresponding to a total store of $2^{20} = 1.049 \times 10^6$ counts. In addition to these 20 counters there are three extra counters which accumulate respectively clock pulses (or number of sampling intervals), clipped photocounts, and unclipped photocounts, in order to determine the mean single-channel counting rates $\langle n \rangle$ and $\langle n_k \rangle$. The contents of the 23 counters were read out continuously at a frequency of 10 kHz and, following digital-to-analog conversion, were displayed on an oscilloscope.

Using a combination of experiment, theory and computer simulation, Hughes *et al.* (1973) have recently made an extensive evaluation of the optimum experimental parameters for a photocount correlation experiment. Although our work was performed before the appearance of this publication, our trial-and-error determination of optimum conditions is in general agreement with Hughes *et al.* Depending on the macromolecular concentration in the solution under study, $\langle n \rangle$ at full laser intensity ranged in our experiments from about 0.1–50 photocounts/sampling time. The correlator could be operated with clipping levels k of 0, 1, 2, and 3, and as suggested by Hughes *et al.*, we generally clipped near the mean counting rate. For concentrated solutions, the laser beam was attenuated until $\langle n \rangle < 3.5$. Optimum could be rapidly selected with the aid of the oscilloscope display. For $\langle n \rangle \gg k$, the shift register contains mostly 1's and there will be a rapid correlation counting rate, but little dependence of c_m on m. For $\langle n \rangle \ll k$ the register contains mostly 0's and the displayed correlation counting rate is extremely low.

We generally chose the clock interval T such that the 20 correlator channels spanned 3 or 4 time constants of $|g(m\mathrm{T})|^2$, *i.e.*, such that $M\mathrm{T} = 1.5$–2 (*cf.* Hughes *et al.*, 1973). The total number of samples N was usually about 10^6; for $\langle n_k \rangle \simeq 0.5$ and $\langle n \rangle \simeq 1$, this corresponds to $c_m \simeq 10^6$. Total experimental run times were frequently as short as 30 sec, but often ranged up to several minutes.

After completion of a run, the counter contents and appropriate switch settings were transferred directly to an IBM 2741 typewriter terminal connected to a time-shared computing system, allowing comprehensive data analysis to be performed rapidly and conveniently.

Data Reduction I

Least-Squares Data Analysis

The basic equation for data analysis is eq 8 relating the average correlation counter contents, $\langle c_m \rangle$, and average

[2] The dark count rate of the uncooled PMT was less than 30/sec.

single-channel counting rates, $\langle n \rangle$ and $\langle n_k \rangle$, to the desired decay rate Γ. However, due to the random natures of both the intensity fluctuations and the photodetection process, one only obtains approximations of above quantities from any realistic experiment. We can define a set of experimental "signals" by

$$S_m = \frac{c_m}{N\langle n \rangle_{ex} \langle n_k \rangle_{ex}} - 1 \tag{10}$$

where

$$\langle n \rangle_{ex} = \sum_{i=1}^{N} n(t_i)/N$$

and

$$\langle n_k \rangle_{ex} = \sum_{i=1}^{N} n_k(t_i)/N \tag{11}$$

which must be fit to the function $\gamma \exp(-2\Gamma m\mathbf{T})$ (eq 8), in order to obtain an experimental value for Γ.

In practice, it is more convenient to fit $\ln S_m$ to the straight line $(\ln \gamma - 2\Gamma m\mathbf{T})$ than to fit S_m directly to an exponential, since in the first case the least-squares problem can be solved analytically; in the other, an iterative procedure is necessary (see, for example, Guest, 1961). Thus data analysis consists of a two parameter (γ and Γ) minimization of the sum

$$\sum_{m=1}^{M} (\ln S_m - \ln \gamma + 2\Gamma m\mathbf{T})^2 \chi_m \tag{12}$$

where the χ_m are weight factors. We assume, as is usual in least-squares analysis (see, for example Guest, 1961; see, also, Jakeman *et al.*, 1971a), that the best fit (best in the sense that the variance of Γ is a minimum) is obtained by taking the χ_m to be inversely proportional to the variance in the measured quantity, *i.e.*

$$\chi_m \propto \frac{1}{\langle(\delta \ln S_m)^2\rangle} \simeq \frac{S_m^2}{\langle(\delta S_m)^2\rangle} \tag{13}$$

where $\langle(\delta S_m)^2\rangle$ is the expected variance in S_m.

Our analysis of computer simulated data has shown that the variance of the estimated value of Γ calculated with the correct weighting factors is smaller by a factor of $\sim$15 than that calculated with uniform weighting. One can, however, neglect the relatively weak m dependence of $\langle(\delta S_m)^2\rangle$ (as suggested by Jakeman *et al.*, 1971a), and use

$$\chi_m \propto S_m^2 \tag{14}$$

Data Reduction II

Detection and Characterization of Nonexponential Correlation Functions

In the discussion so far, we have assumed that the measured signals S_m should have an exponential form $\gamma \exp(-2\Gamma m\mathbf{T})$. In practice, departures from single-exponential behavior result from a variety of effects, such as sample polydispersity, molecular rotation or flexation, dust in the sample, or the detection of stray laser light.[3] We have developed a general formalism which provides a test of how well the actual measured signal conforms to a single exponential, and which can also be used to characterize quantitatively a broad class of nonexponential correlation functions. The formalism is similar to those which have been used to analyze the equilibrium sedimentation of solutions containing several species of macromolecules (Yphantis, 1964), and to analyze chemical relaxation in the presence of several relaxation mechanisms (Schwarz, 1968). The frequency domain analog of the procedure has also been used by Schaefer *et al.* (1971), to treat light scattering from solutions of tobacco mosaic virus, where rotational effects make a significant contribution to the spectrum. A more formal descrition of this approach has appeared elsewhere (Koppel, 1972).

For simplicity, let us first consider macromolecular polydispersity, bearing in mind however that the principles described can equally well be applied to other effects. The square root of the measured signals gives an experimental estimate of $\gamma^{1/2}|g(\tau)|$, the electric field correlation function of the scattered light. For a noninteracting system of polydisperse macromolecules, $|g(\tau)|$ can be written as

$$|g(\tau)| = \int_0^\infty G(\Gamma) \exp(-\Gamma\tau) d\Gamma \tag{15}$$

where $G(\Gamma)$ is the normalized distribution of decay rates

$$G(\Gamma) = \Sigma\langle I_j \rangle \delta(\Gamma - \Gamma_j)/\Sigma\langle I_j \rangle \tag{16}$$

and $\langle I_j \rangle$ is the mean intensity of light scattered by species j. Here δ is the Dirac δ function (*cf.* Margenau and Murphy, 1956). From eq 15, it can be seen that the straightforward approach to the problem of polydispersity would be Laplace inversion of the experimental estimate of $|g(\tau)|$ to yield $G(\Gamma)$. Unfortunately it turns out that this procedure is extremely sensitive to unavoidable statistical errors in the data, and, in general, data of unattainably high precision are needed to perform this inversion to an acceptable degree of accuracy. This insensitivity to sample polydispersity will be illustrated with numerical examples below.

Our formalism recognizes this insensitivity yet, it appears, yields all the meaningful information about $G(\Gamma)$ obtainable from the data.

In eq 15, we expand $\exp(-\Gamma\tau)$ about $\exp(-\bar{\Gamma}\tau)$, where $\bar{\Gamma}$, the mean value of $G(\Gamma)$ is given by

$$\bar{\Gamma} = \int G(\Gamma)\Gamma d\Gamma \tag{17}$$

Then

$$\begin{aligned} |g(\tau)| &= \exp(-\bar{\Gamma}\tau) \int G(\Gamma) \exp((\bar{\Gamma} - \Gamma)\tau) d\Gamma \\ &= \exp(-\bar{\Gamma}\tau) \sum_{r=0}^{\infty} (-1)^r \mu_r/r! \end{aligned} \tag{18}$$

Here μ_r is the rth moment about the mean of $G(\Gamma)$

$$\mu_r = \int G(\Gamma)(\Gamma - \bar{\Gamma})^r d\Gamma \tag{19}$$

It is further convenient to take and expand the logarithm of eq 18

$$\ln(\gamma^{1/2}|g(\tau)|) = \frac{\ln \gamma}{2} - \bar{\Gamma}\tau + \frac{\mu_2(\bar{\Gamma}\tau)^2}{2!\bar{\Gamma}^2} - \frac{\mu_3(\bar{\Gamma}\tau)^3}{3!\bar{\Gamma}^3} + \frac{(\mu_4 - 3\mu_2)(\bar{\Gamma}\tau)^4}{4!\bar{\Gamma}^4} + \cdots \tag{20}$$

Equation 20 is exact provided all the terms are kept.[4] The power of this approach derives from the fact that, for many $G(\Gamma)$'s of interest, the terms in eq 20 fall off rapidly with in-

[3] A fluctuating incident laser intensity is another potential source of error (see, for example, Cummins and Swinney, 1970). In intensity autocorrelation, however, the relative magnitude of this effect is of the order of the square of the fractional intensity change, and can be made negligibly small with ordinary stabilization circuitry.

[4] We note that, formally, $\ln|g(\tau)|$ is the cumulant generating function for $G(\Gamma)$ (see, for example, Kendall, 1948, and Koppel, 1972), and that the coefficient of $(-\tau)^n/n!$ in eq 20 is in fact the nth cumulant of (Γ).

creasing order in $\bar{\Gamma}\tau$. Thus for a sample described by a relatively narrow $G(\Gamma)$, experimental data can be well represented by just the first three terms of eq 20 (see examples below), and it is seldom necessary to use more than four terms of eq 20.

Two approaches can be followed when analyzing data according to eq 20. One can simply fit $\ln S_m^{1/2}$ to the appropriate polynomial in τ (Koppel, 1972). Alternatively, successive numerical derivatives of the data can be performed, *e.g.*

$$\frac{d \ln}{d\tau} |g(\tau)| = -\bar{\Gamma} + \mu_2\tau - \frac{1}{2!}\mu_3\tau^2 + \cdots \quad (21)$$

Differentiation provides a useful visual display of data; if the data can truly be described by a single exponential, only $\bar{\Gamma}$ will be nonzero, and the first numerical derivative plotted as a function of τ will be a horizontal straight line. On the other hand, for multiexponential data, this plot will show a definite trend away from the horizontal, with a gradient, which, for $\tau \rightarrow 0$, will give μ_2. (An example of such a plot is in the Results section.) We have applied this formal procedure routinely to all data, as a test of single exponentially. The intercept of the first derivative plot is an estimate, $\hat{\Gamma}$, of $\bar{\Gamma}$, and the slope obtained by force-fitting this plot to a straight line gives an estimate $\hat{\mu}_2$ of μ_2. We then define a quality parameter Q by

$$Q = \hat{\mu}_2/(\hat{\Gamma})^2 \quad (22)$$

Only if Q is zero to within experimental error can the data be said to conform to a single exponential. A roughly equivalent approach, which is simpler in practice, is to determine Q by fitting $\ln S_m^{1/2}$ directly to $(1/2) \ln \gamma - \hat{\Gamma}\tau + (1/2!)\hat{\mu}_2\tau^2$.

We now briefly illustrate these ideas with two experimental examples. Consider two two-component solutions which can be described in the distribution functions

$$G_1(\Gamma) = 0.5\delta(\Gamma - 0.8\Gamma_0) + 0.5\delta(\Gamma - 1.2\Gamma_0) \quad (23)$$

and

$$G_2(\Gamma) = 0.8\delta(\Gamma - 0.9\Gamma_0) + 0.2\delta(\Gamma - 1.4\Gamma_0) \quad (24)$$

For both these distributions the decay rates of the two components are roughly in the ratio 1.5:1. Such a difference in diffusion coefficients might be expected, for example, between the monomer and dimer of some macromolecular species. In Table I we list the first few moments of these distributions, calculated directly from eq 17, 19, 23, and 24. It can be seen that the distributions were chosen to have the same mean and variance and that they differ only in the higher moments. Also listed are the quantities Γ_{SE}, the decay rates found by force fitting exact computer-simulated data to single exponentials over the range $0 \leq \Gamma_0\tau \leq 2$, and the values of Q found for these data using the procedure outlined above. For G_1, $Q \simeq \mu_2/\Gamma^2$ indicating that the data are well described by just the first three terms of eq 20 over this range in τ. For G_2 the higher order terms make a larger, but still relatively small, contribution.

To evaluate the effect of experimental errors on our procedure we have analyzed about 10 sets of computer-simulated data for both G_1 and G_2, including realistic statistical spread. We took

$$\langle c_m \rangle = 10^6(1 + |g(\tau)|^2)$$

and assumed the c_m to be Poisson distributed with mean $\langle c_m \rangle$, as expected for low counting rates ($\langle n \rangle < 0.1$, see, for example, Jakeman *et al.*, 1971a). Bearing in mind that our correlation counters have a capacity of about 10^6 counts,

TABLE I: Normalized Moments of the Distributions $G_1(\Gamma)$ and $G_2(\Gamma)$.

	$G_1(\Gamma)$	$G_2(\Gamma)$
	Theoretical	
$\bar{\Gamma}$	Γ_0	Γ_0
$\mu_2/\bar{\Gamma}^2$	0.04	0.04
$\mu_3/\bar{\Gamma}^3$	0	0.012
$\mu_4/\bar{\Gamma}^4$	0.0016	0.0052
$\bar{\Gamma}_{SE}$	$0.978\Gamma_0$	$0.979\Gamma_0$
Q	0.0391	0.0321
	Computer Simulated Data with Errors	
Γ_{SE}/Γ_0	0.975 ± 0.002	0.978 ± 0.002
$\hat{\Gamma}/\Gamma_0$	0.999 ± 0.008	0.995 ± 0.008
Q	0.040 ± 0.010	0.037 ± 0.010

the data generated in this way can be expected to be a little more precise than the best data attainable in a single run with our apparatus. The results of these analyses are also given in Table I.

Several conclusions can be drawn. Thus, even with data of quite high precision, it is impossible by intensity fluctuation spectroscopy (IFS) to distinguish between the distributions G_1 and G_2. IFS is in fact quite insensitive to sample polydispersity. One can obtain a mean decay rate with a fair degree of accuracy and the second moment μ_2 with lower accuracy. However, a knowledge of just two parameters is insufficient for a complete characterization of $G(\Gamma)$.

On the positive side, however, is the fact that $\bar{\Gamma}$ and μ_2 are well-defined quantities. In many cases of interest, $\bar{\Gamma}$ can be related to a useful average diffusion coefficient. From eq 16 and 17, assuming $\Gamma_j = D_jK^2$, we get

$$\bar{D} \equiv \bar{\Gamma}/K^2 = \sum_j \langle I_j \rangle D_j / \sum_j \langle I_j \rangle$$

For solutions of macromolecules small compared to $1/K$, with equal polarizability per unit mass

$$\langle I_j \rangle \propto N_j M_j^2$$

where M_j is the molecular weight of species j and N_j is its number density. Then

$$\bar{D} = \Sigma N_j M_j^2 D_j / \Sigma N_j M_j^2$$

the so-called z-average diffusion coefficient $\bar{D}_z$ (*cf.* Tanford, 1961). When $\bar{D}_z$ is combined in the Svedberg equation with the weight-average sedimentation constant, readily obtained for a polydisperse system by appropriate analysis of centrifuge data (Tanford, 1961), the weight-average molecular weight is obtained (Kinnel, 1959; see, also, Gibbons, 1971). This fact allows, for the first time, the sedimentation–diffusion method to be used to determine a meaningful molecular weight average of a polydisperse system. (It should be mentioned that if $S_m^{1/2}$ is force fitted to a single exponential, the decay rate so obtained has no formal significance.)

As mentioned at the start of this section, various spurious effects can also cause a departure of the observed correlation function from a single exponential. The consequences of dust in the sample and the detection of stray laser light are essentially the same—the apparent presence of slower decaying components in the measured signal, S_m. With stray laser light, one has a heterodyne term with a decay rate one-half that of the principle homodyne term. With dust in the sample,

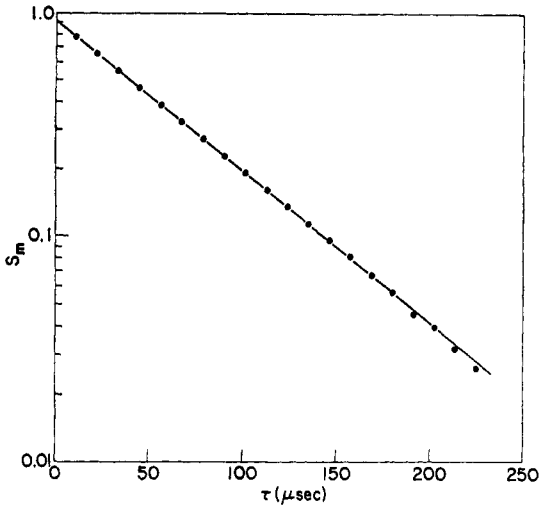

FIGURE 4: Correlator output for sample of R17 virus.

one has, in addition, a dust phase fluctuation term (with decay rate $\propto D$ dust), and a dust "number fluctuation" term (Schaefer and Berne, 1972), which, for the usual range of correlation delay times, appears as an additional constant background.

We have analyzed these effects with computer simulated data and have found that, to first order

$$(\Gamma - \Gamma_{SE})/\Gamma \approx Q$$

A similar conclusion was reached by Ford (1972). The typical uncertainty in Q for a single run with our apparatus was ± 0.02. Thus if, when studying a monodisperse system, we discard data for which $|Q| > 0.02$, the presence of dust or stray laser light should cause an uncertainty in Γ of at most 2%.

Sources and Magnitude of Error in an Absolute Determination of D

To make unambiguous comparisons between experiments conducted under different conditions, it is traditional to correct the diffusion coefficient $D_{T,S}$, obtained at arbitrary temperature T (in °C) in arbitrary solvent S, to standard conditions: 20° and water as solvent. $D_{T,S}$ is in general given by (see, for example, Tanford, 1961)

$$D_{T,S} = \frac{k(T + T_0)}{6\pi\eta_{T,S}R_h(f/f_0)} \qquad (25)$$

where $T_0 = 273.16$°K, $\eta_{T,S}$ is the viscosity of the solvent at temperature T, f/f_0 is a shape factor (= 1, for a sphere), k is Boltzmann's constant, and R_h is the radius of the equivalent

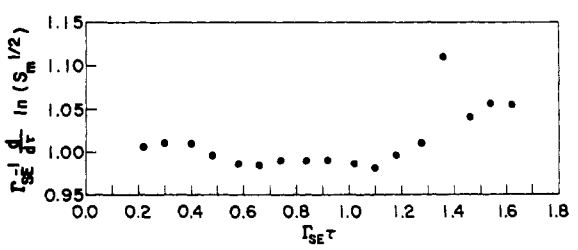

FIGURE 5: First derivative with respect to delay time of plot of Figure 4.

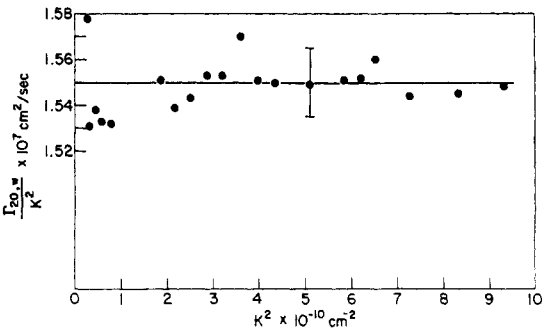

FIGURE 6: Dependence of decay rate $\Gamma_{20,w}/K^2$ on K^2 for sample of R17 virus.

sphere (the sphere with the volume of the hydrated particle). For a system for which $\Gamma = DK^2$, then

$$D_{20,w} = \frac{\Gamma_{T,S}}{K^2} \times \frac{\eta_{T,S}}{\eta_{20,w}} \times \frac{T_0 + 20°}{T_0 + T} \qquad (26)$$

In what follows we consider, in turn, the effect on $D_{20,w}$ of errors in $\Gamma_{T,S}$, K^2, $\eta_{T,S}$, and T.

Errors in $\Gamma_{T,S}$. The question of estimating the random statistical error in the measurement of Γ has been discussed by several authors. The treatment most directly applicable to our work is that of Hughes *et al.*, 1973 (see also Jakeman *et al.*, 1971a). From this work we find that the predicted error in Γ for a single typical experimental run with our apparatus is about 1%. Indeed we found that the values of Γ obtained from several runs under identical experimental conditions generally fluctuated within this range.

The remaining potential source of systematic error in Γ is nonexponentiality of the photocount correlation function which was discussed in the previous section. For all of the data presented in the accompanying paper (Camerini-Otero *et al.*, 1974), the quality factor Q was zero to within experimental error (<0.02). Occasionally, at low virus concentration, nonzero values of Q were obtained, indicating the presence of dust, which could usually be removed by further filtration of the sample. Independent measurements on the viruses of interest by electron microscopy and ultracentrifugation have shown the preparations to be monodisperse (Camerini-Otero *et al.*, 1974). Thus we expect no nonexponentiality from this source. We usually combined the results of about six independent runs, thereby reducing the error in Γ by a factor of about $(6)^{1/2}$ (see the following paper, Camerini-Otero *et al.*, 1974).

Error in K. The magnitude of K is given by eq 7. The laser wavelength *in vacuo*, λ_0, is known with negligible error. The solution refractive index n_0 was obtained to better than 1 part in 10^3 by measuring the angle of minimum deviation of the laser beam through a corner of the square sample cell. This value of n was also used to make the small refraction correction to the scattering angle required when using a square sample cell, if the refractive indices of the sample and bath water are significantly different. The main source of error in K is error in the measurement of the scattering angle. Differentiating the square of eq 7 with respect to θ gives

$$\Delta(K^2)/K^2 \simeq (\Delta\theta/57) \tan(\theta/2) \qquad (27)$$

where $\Delta\theta$ is in degrees. For our apparatus $\Delta\theta \lesssim 0.3°$. This gives $\Delta(K^2)/K^2 < 1.3\%$ for $\theta = 45°$, $\Delta(K^2)/K^2 < 0.5\%$ for $\theta = 90°$, and $\Delta(K^2)/K^2 < 0.1\%$ for $\theta > 160°$.

Error in $\eta_{T,S}$ *and* T. The solvent viscosity $\eta_{T,S}$ at a given

temperature T can be measured in a simple viscometer to better than 0.5%. For water, η varies by about 10% in the temperature range 20–25°. Since the temperature was known with an absolute accuracy of about 0.1° the uncertainty in $\eta_{T,S}$ was about 0.2%. The error in $(T_0 + 20)/(T_0 + T)$ due to temperature uncertainty was negligible.

We can now combine these errors to estimate the error in a measurement of $D_{20,w}$, in the usual fashion by differentiating eq 27 with respect to Γ, K^2, and $\eta_{T,S}$

$$\frac{\Delta D_{20,w}}{D_{20,w}} = \left[\left(\frac{\Delta\Gamma}{\Gamma}\right)^2 + \left(\frac{\Delta K^2}{K_2}\right)^2 + \left(\frac{\Delta\eta_{T,S}}{\eta_{T,S}}\right)^2\right]^{1/2} \quad (28)$$

Combining the errors according to eq 28, $\Delta D_{20,w}/D_{20,w} \simeq 1\%$.

By using longer experimental run times and more precise measurements of θ and $\eta_{T,S}$, it should be possible, with existing apparatus, to measure $D_{20,w}$ with 0.1% accuracy. Such accuracy could be useful in detecting the small conformational changes that enzymes and proteins are believed to undergo when performing their biological function. They would complement related information presently attainable by the technique of difference sedimentation (Kirschner and Schachman, 1971).

Results

In this section we present some data to illustrate the typical performance of the apparatus. Figure 4 shows a semilog plot of experimental data for a solution of R17 virus in 0.015 M NaCl, at a viral concentration of 1.68 mg/ml (see also accompanying paper, Camerini-Otero *et al.*, 1974). Experimental parameters for this run were as follows: temperature, T, 25°, scattering angle, θ, 90°; sample time, T, 11.25 μsec; mean photocount rate, $\langle n\rangle$, 0.66; clipping level, k, 1; total number of sampling intervals, N, 6×10^6; run time, NT, 67 sec; Analysis of the data gave T_c ($\equiv 1/\Gamma_{SE}$) = 128.0 μsec. Thus the number of coherence times spanned by the correlator, $M\Gamma$T, was about 1.75.

It can be seen from Figure 4 that ln S_m appears to be well fitted by a straight line. In Figure 5 we plot the first numerical derivative with respect to τ of ln $S_m^{1/2}$ for the data of Figure 4. The differentiation was performed by taking all possible sets of four consecutive data points and fitting ln $S_m^{1/2}$ to a straight line for each set. The slopes so obtained are estimates of $(d/d\tau)$ ln $S_m^{1/2}$ at the mean delay times of the four-point segments. It can be seen from Figure 4 that ln $S_m^{1/2}$ appears to be well fitted by a straight line. There is no obvious trend evident in this plot, and the quality parameter Q was calculated to be 0.017 ± 0.02, giving no evidence of nonexponential behavior.

As a demonstration of the quality of the samples, and the accuracy of alignment of the photometer, we give, in Figure 6, a plot of $\Gamma_{20,w}/K^2$ *vs.* K^2 for a sample of R17 virus in 0.15 M NaCl, at a viral concentration of 0.324 mg/ml. The scattering vector, K, was varied both by changing θ, in the range $20° < \theta < 120°$, and by using three laser wavelengths, 6471, 5682, and 4762 Å. Since we expect $\Gamma = DK^2$, this plot should theoretically be a horizontal straight line at $\Gamma/K^2 = D_{20,w}$. This prediction is, to within experimental error, fulfilled by the data. The line in Figure 6 is the average of the data points, and the error bar indicates 1% error. With the exception of one point, all the data for $K^2 > 1.5 \times 10^{10}$ cm^{-2} lie within this error. For $K^2 < 1.5 \times 10^{10}$ cm^{-2}, the scatter in the data is greater, since Γ becomes small as $K \to 0$, and the expected error in the data is proportional to $\Gamma^{-1/2}$ (Jakeman *et al.*, 1971a). For this range of K, we did not fully compensate for the loss of precision due to small Γ with longer experimental run times.

We also performed runs at $\theta = \pm 25°$. The average values of Γ obtained for these two angles differed by less than 2%, indicating that alignment error was less than 0.15° for this set of data.

Each point in Figure 6 is the average of two or three runs. An indication of the speed of multichannel digital correlation, when combined with a versatile photometer and on-line data analysis, is the fact that the 50 or so independent diffusion coefficient measurements comprising Figure 6 were performed and analyzed in a single afternoon.

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Intensity Fluctuation Spectroscopy of Laser Light Scattered by Solutions of Spherical Viruses: R17, Qβ, BSV, PM2, and T7. II. Diffusion Coefficients, Molecular Weights, Solvation, and Particle Dimensions†

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ABSTRACT: The diffusion coefficients (D) of several spherical viruses have been determined by the method of intensity fluctuation spectroscopy. The values for $D^0_{20,w}$ for bacteriophages R17, Qβ, PM2, and T7 are (in units of 10^{-7} cm^2 sec^{-1}): 1.534 ± 0.015, 1.423 ± 0.014, 0.650 ± 0.007, and 0.644 ± 0.007, respectively; that for the plant virus, tomato bushy stunt virus (BSV), is 1.246 ± 0.012. The molecular weights were calculated from these values of D combined with literature values for the sedimentation coefficients (s) and values for the apparent specific volumes determined by us (R17, Qβ, and PM2) or obtained from the literature (BSV and T7). The calculated molecular weights for R17, Qβ, BSV, PM2, and T7 are (in millions of daltons): 4.02 ± 0.17, 4.55 ± 0.16, 8.81 ± 0.17, 47.9 ± 1.7, and 50.9 ± 1.1, respectively. These values are in good agreement with literature values and those estimated from composition. Using the Stokes–Einstein equation, we have calculated hydrodynamic radii, R_h, for these viruses from our values of $D^0_{20,w}$. These radii are 140 ± 2, 151 ± 2, 172 ± 2, 330 ± 3, and 333 ± 3 Å for R17, Qβ, BSV, PM2, and T7, respectively, and are in agreement with the dimensions obtained by such techniques as low-angle X-ray scattering, light scattering, and electron microscopy. Values for the solvation of these viruses, derived from the data, are (in cm^3 of solvent/g of virus): 1.02 ± 0.09, 1.22 ± 0.08, 0.75 ± 0.04, 1.11 ± 0.08, and 1.18 ± 0.06, in the same order as above. The diffusion coefficients for all the viruses were studied as functions of virus concentration. In addition, D for R17 and PM2 was studied as a function of ionic strength, and D for R17 was also measured as a function of temperature and pH.

The method of intensity fluctuation spectroscopy of scattered coherent (laser) light (Pecora, 1964; Dubin *et al.*, 1967, 1970; Cummins *et al.*, 1969; Foord *et al.*, 1970; Pusey *et al.*, 1972, 1974) has been used to determine the translational diffusion coefficients[1] of several nearly spherical viruses: the bacteriophages R17, Qβ, PM2, and T7 and the plant virus,

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[1] Throughout we shall assume *translational* diffusion coefficients to be understood.