

Laser Beat Frequency Spectroscopy

Measurements on rotation and translation of biomolecules in solution are accurate and rapid.

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The measurement of static properties such as molecular weights, shapes, and dimensions of macromolecules by light scattering is not new. Laser beat frequency spectroscopy provides information on the kinetic properties as well; for example, translational and rotational diffusion coefficients. Whereas light scattering using conventional light sources relies on the angular dependence of the intensity and polarization, laser beat frequency spectroscopy permits examination of the spectral distribution of the scattered radiation as well. The scattered laser light is Doppler-broadened in frequency by the random, Brownian, motion of the molecules.

This relatively new technique, which has been feasible only since the advent of the laser, provides a rapid and accurate means of measuring kinetic properties from which size and conformational changes of the biomolecules in solution can be inferred. The technique may be applied to molecules as small as 50 angstroms and is more rapid than conventional light scattering techniques. A spectrum may be obtained in less than 10 minutes under favorable circumstances.

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Theory: Translational Diffusion

Radiation is not scattered from an optically homogeneous medium. If, however, random molecular motions produce local fluctuations in the dielectric constant, one will observe light scattered at directions other than the incident. If the fluctuations are dependent on time, then the scattered light will be Doppler-shifted. The spectral distribution and angular dependence of the scattered light contain information on the spatial and temporal variations of the fluctuations in dielectric constant. Actually they give, in effect, a complete Fourier analysis of the fluctuations in space and time.

We present a brief, simple review of the relevant theory; detailed treatments are given by Mountain (1), Komarov and Fisher (2), and by Pecora (3, 4). We restrict ourselves to the case where the local fluctuations in dielectric constant arise solely from the fluctuations in concentration. The contributions to the spectrum from isentropic density fluctuations—that is, thermally excited sound waves—and nonpropagating entropy fluctuations are ignored. This assumption is, in general, valid for the scattering from macromolecules; however, the validity must be carefully examined for each individual system to be studied.

In order to describe the scattering of light from a fluid, such as a solution of macromolecules, we subdivide the illuminated volume into small elements, compute the field scattered from one element, and then add up the contributions from all the elements; at the same time we take into account the phase relations of the light scattered from different parts of the illuminated volume. This procedure is the same as that used by Gingrich (5) to describe x-ray scattering from liquids.

The scattering from a single fluid element is shown in Fig. 1. Here $\hat{u}_0$ and $\hat{u}_s$ are unit vectors in the direction of the incident and scattered beams respectively. The phase of the light reaching the detector will be measured with reference to the phase of the radiation that goes to a reference point and then onto the detector. The difference in path lengths between the beam scattered from the j^{th} fluid element and the reference path is just

$$\mathbf{r}_j \cdot \hat{u}_s - \mathbf{r}_j \cdot \hat{u}_0,$$

where $\mathbf{r}_j$ is the distance between the volume element and reference point. If one assumes that the wavelength changes caused by the Doppler effect are small, then the difference in radians between the two paths is simply

$$2\pi \left[\frac{\mathbf{r}_j \cdot (\hat{u}_s - \hat{u}_0)}{\lambda} \right]$$

which can be written in terms of the wave vectors of the incident and scattered beams as

$$\mathbf{r}_j \cdot (\mathbf{k}_s - \mathbf{k}_0).$$

It is customary to define the scattering vector $\mathbf{K}$ as $\mathbf{K} = \mathbf{k}_s - \mathbf{k}_0$, so that the difference in path length becomes $\mathbf{K} \cdot \mathbf{r}_j$ radians. The path length in radians from the reference point to the detector is $\mathbf{k}_s \cdot \mathbf{R}$. The phase of light scattered from element j will differ from this by $-\mathbf{K} \cdot \mathbf{r}_j$ radians.

From the theory of Rayleigh scattering (6) the intensity of the field $E_{j(t)}$ scattered into a point at a distance R from the scattering center is

$$E_{j(t)} = - \left[\frac{\pi E_0 \sin \phi}{R \lambda^2} \right] \times V_j \delta \epsilon_{j(t)} \exp i (\mathbf{k}_s \cdot \mathbf{R} - \mathbf{K} \cdot \mathbf{r}_j - \omega_0 t) \quad (1)$$

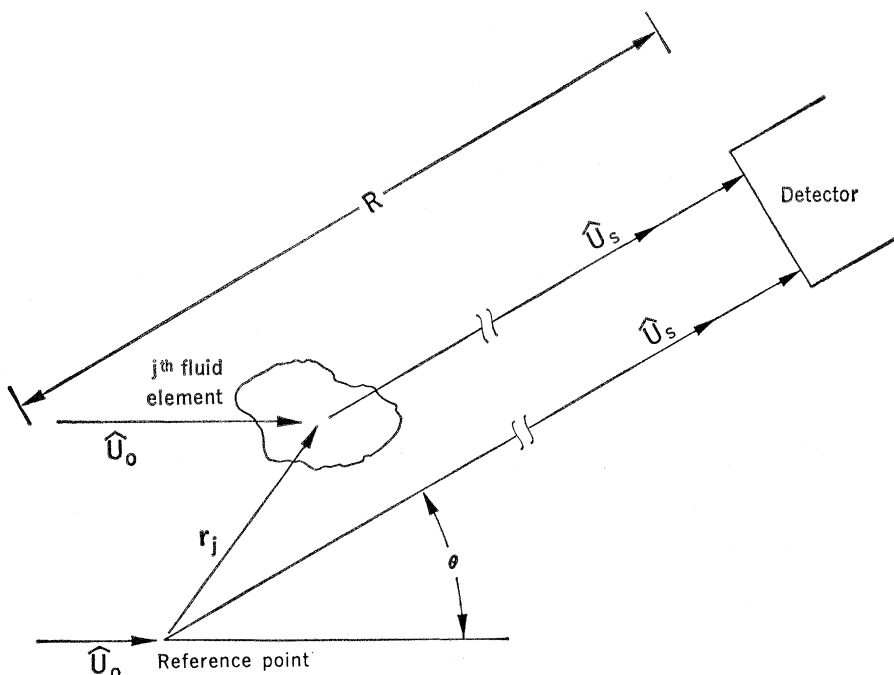


Fig. 1. Light scattering from a single fluid element.

where $\delta\epsilon_j(t)$ is the deviation of the dielectric constant of the fluid element j from the average value for the solution; V_j is the volume of the element; ϕ is the angle between the scattered wave vector and the electric field of the incident radiation; and ω_0 is the angular frequency of the incident light.

The total field scattered into the detector is the sum of the contributions from all the fluid elements:

$$E(t) = \zeta_0 [\exp(-i\omega_0 t)] \sum V_j \delta\epsilon_j(t) \times \exp(-i\mathbf{K} \cdot \mathbf{r}_j) \quad (2)$$

where ζ_0 replaces the constant terms. The summation may be replaced by an integration over the illuminated volume; therefore

$$E(t) = \zeta_0 [\exp(-i\omega_0 t)] \int \int \int \delta\epsilon(r, t) \times [\exp(-i\mathbf{K} \cdot \mathbf{r})] d^3r \quad (3)$$

Equation 3 has the form of a three-dimensional spatial Fourier transform, except that the limits on the integration run only over the illuminated volume rather than over the whole of space. However, for the scattering angles normally employed, say 45° , with visible radiation, the wave vector $\mathbf{K}$ is of the order of 10^5 cm^{-1} with the result that $\mathbf{K} \cdot \mathbf{r}$ goes through many radians even though the dimensions of the illuminated volume may be as little as 10^{-3} centimeter in diameter. Consequently, the integral may be regarded as the Fourier transform and we have directly

$$E_{(\mathbf{K}, t)} = \zeta_0 [\exp(-i\omega_0 t)] \times (2\pi)^{1/2} \delta\epsilon_{(\mathbf{K}, t)} \quad (4)$$

Equation 4 is simply a statement that the scattered field with a scattering vector $\mathbf{K}$ comes from the spatial Fourier component of the dielectric constant fluctuation with a wave vector $\mathbf{K}$.

Classically, this corresponds to constructive interference, or Bragg law diffraction, from a sinusoidal grating with a wavelength $\Lambda = 2\pi/K$. Quantum mechanically, Eq. 4 represents the condition for the conservation of momentum.

Choice of the scattering angle θ and the wavelength of the incident radiation λ fixes the spatial Fourier component from which the scattering is being observed. For small Doppler shifts $k_0 \cong k_s$, and the relation between K and θ is given by the Bragg condition

$$K = 2 k_0 \sin(\theta/2) \quad (5)$$

where θ is the angle between $\mathbf{k}_s$ and $\mathbf{k}_0$.

The spectral composition of the scattered field may be obtained by taking the Fourier transform of Eq. 4 into the frequency domain. Before this transformation can be made, however, an expression for the time-dependent dielectric constant fluctuations $\delta\epsilon_{(\mathbf{K}, t)}$ must be derived. To this end we introduce two assumptions: first, that the deviation in local dielectric constant is caused solely by deviations in the concentration, C

$$\delta\epsilon_{(\mathbf{K}, t)} = (\partial\epsilon/\partial C) \delta C_{(\mathbf{K}, t)} \quad (6)$$

Second, we assume that the microscopic concentration fluctuations obey, on the average, the macroscopic trans-

lational diffusion equation, Fick's law. Rotational diffusion is ignored for the moment

$$\frac{d\delta C_{(\mathbf{r}, t)}}{dt} = D \nabla^2 \delta C_{(\mathbf{r}, t)} \quad (7)$$

where D is the translational diffusion coefficient, assumed to be independent of concentration. Following the procedure suggested by Landau and Placzek (7), we take the spatial Fourier transform of Eq. 7.

$$\frac{d\delta C_{(\mathbf{K}, t)}}{dt} = -K^2 D \delta C_{(\mathbf{K}, t)} \quad (8)$$

Solved by inspection, Eq. 8 gives

$$\delta C_{(\mathbf{K}, t)} = \delta C_{(\mathbf{K}, 0)} \exp(-K^2 D t) \quad (9)$$

Using Eqs. 4, 6, and 9, we obtain

$$E_{(\mathbf{K}, t)} = (2\pi)^{1/2} \zeta_0 V (\partial\epsilon/\partial C) \times \delta C_{(\mathbf{K}, 0)} \exp[-(K^2 D + i\omega_0)t] \quad (10)$$

The spectrum of the scattered light can be obtained by taking the time-to-frequency transform of Eq. 10 to obtain $E_{(\mathbf{K}, \omega)}$. The energy spectrum of the scattered light is

$$(c/2\pi) [E_{(\mathbf{K}, \omega)} E_{(\mathbf{K}, \omega)}^*]$$

This quantity is the total energy contained in the signal between ω and $\omega + d\omega$. Detection schemes normally measure the average of this quantity divided by the time of measurement T . The quantity measured, referred to as the power spectrum $S_{(\mathbf{K}, \omega)}$, is

$$S_{(\mathbf{K}, \omega)} = \frac{\zeta_0^2 c (\partial\epsilon/\partial C)^2 \langle \delta C_{(\mathbf{K})}^2 \rangle}{2\pi T} \times \left[\frac{K^2 D}{(K^2 D)^2 + (\omega - \omega_0)^2} \right] \quad (11)$$

where $\langle \delta C_{(\mathbf{K})}^2 \rangle$ denotes the average of the square of the concentration fluctuations with a wave vector, $\mathbf{K}$. An instructive discussion of radiation detectors is given by Stone (8).

The bracketed term in Eq. 11 shows that the spectrum has a Lorentzian line shape centered about the incident frequency ω_0 and has a characteristic half-width at half-height of $K^2 D$. Since K is known from Eq. 5, the measurement of the linewidth gives, in principle, a measurement of the diffusion coefficient.

To simplify the computation we have considered only the diffusional decay of the Fourier component. In actuality the magnitude of the Fourier component increases and decreases as the molecules undergo their random motions. The magnitude of the scattered field is, therefore, a random variable whose spectral composition is given by the Fourier transform of its autocorrelation function. The results obtained

are identical to those presented above.

Substituting reasonable values for K and D in Eq. 11, we find that the spectral width is in the range 10^2 to 10^4 hertz. Since visible radiation has a frequency of the order of 5×10^{14} hertz, we require a spectrometer with a resolving power of the order of 10^{13} compared to the resolving power of 10^9 available with the best Fabry-Perot optical spectrometers. Laser beat frequency spectroscopy provides such a resolving power.

Rotational Diffusion

In Eq. 7 we have assumed that the concentration fluctuation can decay only by translational diffusion. This is a valid assumption for rigid, isotropic molecules but is not necessarily true for anisotropic molecules, such as rigid rods or random coils (3).

At small scattering angles, where the fluctuation wavelength Λ is much greater than any of the molecular dimensions, the contribution to the spectrum from rotational diffusion is small and the observed spectrum is solely from translational diffusion. At large θ , that is small Λ , the contribution to the spectrum from rotational diffusion becomes important for most long-chain molecules and must be considered. Pecora (3, 4) has computed the expected spectrum from both rigid rods and from random coils. For rigid rods, the spectrum is a Lorentzian line with a half-width at half-height of 6Θ , where Θ is the rotational diffusion coefficient. For flexible polymers, the spectrum is a sum of Lorentzians, one for each normal mode of vibration. The rotational diffusion coefficient for tobacco mosaic virus, a frequently studied rod-shaped molecule is 370 sec^{-1} , so that the spectral broadening expected from rotational diffusion is of the same order of magnitude as that from translational diffusion.

Optical Mixing: Beat Frequency

Detection

Laser light sources with their intrinsically narrow bandwidths together with detection by optical mixing enables a spectrometer of the required resolving power to be constructed. The beat frequency spectrometer was first suggested by Forrester and co-workers (9) and was later extensively developed by Ford and Benedek (10) and Lastovka and Benedek (11). The spec-

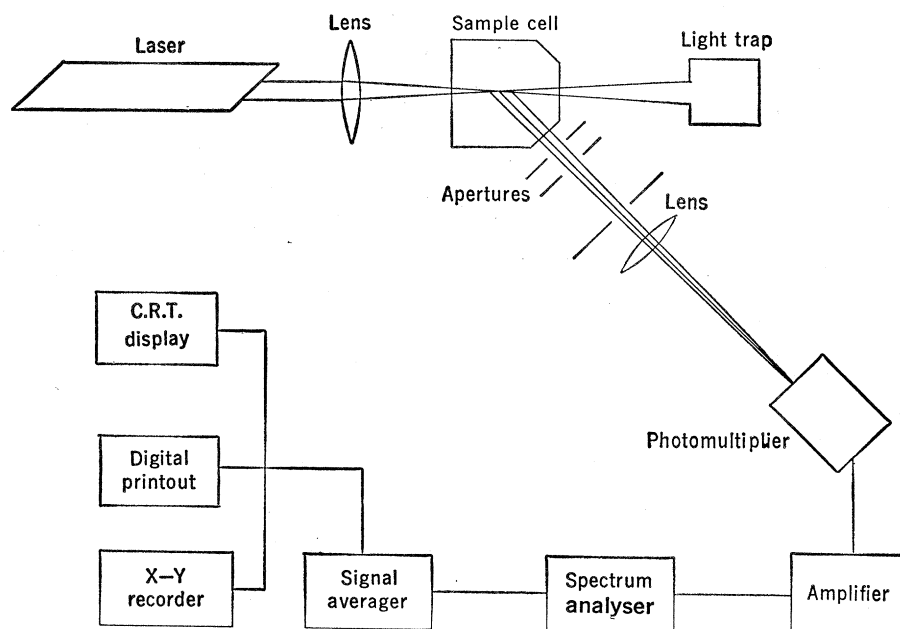


Fig. 2. Schematic diagram of the laser self-beat spectrometer.

trometer is most easily understood by considering the process of optical mixing.

Radiation is incident upon the photocathode of a photomultiplier tube. The photocurrent is proportional to the square of the total incident field; hence, the term "square law" detector

$$i(t) = \beta E^2(t) \quad (12)$$

or in complex notation

$$i(t) = \beta [E(t) E^*(t)] \quad (13)$$

where $E^*(t)$ is the complex conjugate of $E(t)$, and β is a constant. If the input to a square law or nonlinear detector contains two discrete frequencies, ω_1 and ω_2 , the output will be modulated at the sum, $\omega_1 + \omega_2$, and the difference, $\omega_1 - \omega_2$, as well as at the original frequencies. For example, in the case where the incident field is given by

$$E = \xi_1 \cos \omega_1 t + \xi_2 \cos \omega_2 t,$$

substitution in Eq. 12 gives

$$i(t) = \beta [(\xi_1^2/2)(1 + \cos 2\omega_1 t) + (\xi_2^2/2)(1 + \cos 2\omega_2 t) + \xi_1 \xi_2 \cos(\omega_1 + \omega_2)t + \xi_1 \xi_2 \cos(\omega_1 - \omega_2)t] \quad (14)$$

As the photomultiplier tube does not have an unlimited frequency response, the highest frequency that it can follow being limited to around a kilomegahertz, the first three terms in Eq. 14 average out to a d-c component. The fourth term, $\xi_1 \xi_2 \cos(\omega_1 - \omega_2)t$, however, is a low-frequency component and is referred to as the beat frequency.

In the circumstance that either or

both of the electric fields incident on the photomultiplier tube is not a discrete frequency but possesses a spectrum, then the beat mode will not be a single discrete frequency, but it too will exhibit a spectrum. To obtain this spectrum we take the Fourier transform of Eq. 13 into the frequency domain.

In a self-beat experiment, ideally only one beam of light is incident in the photocathode. In our applications, this light is scattered from the molecules in the solution, and the time-dependent scattered field is given by Eq. 10. Substituting into Eq. 13, we have

$$i(t) = 2\pi \beta \xi_0^2 (\partial \epsilon / \partial C)^2 \times (\delta C^2(K)) [\exp(-2K^2 D t)] \quad (15)$$

Taking the Fourier transform into the frequency domain

$$i(\omega) = \sqrt{2\pi} \beta \xi_0^2 (\partial \epsilon / \partial C)^2 (\delta C^2(K)) \times \int_0^\infty \exp(-2K^2 D t) \exp(i\omega t) dt \quad (16)$$

The integration may be taken from zero to infinity rather than over the entire time domain because the experiment is presumed to start (light turned on) at time equal to zero. From Eq. 16 we obtain

$$i(\omega) = \sqrt{2\pi} \beta \xi_0^2 (\partial \epsilon / \partial C)^2 (\delta C^2(K)) \times \left[\frac{1}{i\omega - 2K^2 D} \right] \quad (17)$$

The power spectrum of the photocurrent $S_{i(K,\omega)}$ is proportional to the time average of $i^2(\omega)$ which, from Eq. 17 is

$$S_{i(K,\omega)} \propto \left[\frac{1}{\omega^2 + (2K^2 D)^2} \right] \quad (18)$$

We see from Eq. 18 that $S_i(K, \omega)$ is a Lorentzian curve centered about zero frequency, that is, has its maximum value when ω equals zero, and has a width at half-height of $2K^2D$.

The net result of the self-beating, therefore, is to shift the original spectrum from the frequency of the incident light down to frequencies of the order of the original linewidth. Experimentally, in this frequency range, the transformation of the photocurrent from the time to the frequency domain is performed by a conventional audio spectrum analyzer.

It is instructive to consider what happens when radiation scattered from the solution mixes on the surface of the photocathode with unbroadened radiation from the same laser. This unbroadened radiation may reach the photocathode by scattering from stationary surfaces such as the cell walls. Its time dependence will be purely sinusoidal, that is, $\xi_s \exp(-i\omega_0 t)$.

The total incident field is, therefore,

$$\xi \exp(-K^2Dt - i\omega_0 t) + \xi_s \exp(-i\omega_0 t)$$

Performing the same analysis as given above, one finds that the power spectrum of the photocurrent contains a Lorentzian component with width at half-height of K^2D , centered at zero frequency. This component arises from the mixing of the broadened and unbroadened radiation. Since this component lies on top of the self-beat component in the frequency domain, the presence of the unbroadened light will cause the measured linewidths to be erroneously narrow. Extraneous, unbroadened light must, therefore, be rigorously prevented from falling on the surface of the photomultiplier when a self-beating experiment is performed. In other experiments, it may be desirable to purposely mix the scattered light with unbroadened light, for example, when we deal with very low levels of scattered light or when we measure the frequency shift as well as the width of a spectral component (11).

Description of Spectrometer

The laser self-beat spectrometer which we use (Figs. 2 and 3) is similar to that used by Ford and Benedek (10) and Lastovka and Benedek (11). A Spectra Physics model 125 continuous-wave helium-neon laser provides about 75-milliwatts incident power at 6328 angstroms. A helium-neon laser does

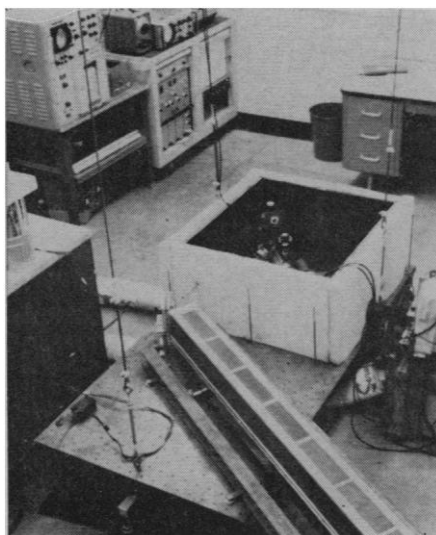


Fig. 3. Spectrometer assembly. In the foreground are the laser and the thermally controlled box containing the optics supported on a machinist's layout table. The cables suspending the table from the vibration isolation mount are visible. In the background are the spectrum analyzer and the signal averager.

not radiate at a single frequency, but in a series of modes. The so-called axial modes are equally spaced about the center frequency of 4.741×10^{14} hertz, and the intermodal spacing is given by $c/2L$, where L is the length of the laser cavity. For the model 125 the cavity length is 183 cm, corresponding to an axial mode separation of 83 megahertz, and around 20 modes are normally detectable. Each of these modes will contribute to the effective electric field incident on the photocathode. The frequency of the beats between the axial modes, however, is much higher than the frequency range of interest in diffusion coefficient measurements and can cause no harm. Transverse, off-axis modes, however, are more closely spaced and may be unstable. A laser used for laser self-beat spectroscopy should resonate only in the axial (TEM_{00}) modes.

The output frequency ω_0 of the laser may drift with time. However, because of the self-beating detection ω_0 does not explicitly appear in the expression for the power spectrum of the photocurrent. It does influence the measured broadening, inasmuch as it affects K^2 . However, a frequency drift of as much as a kilomegahertz corresponds only to a change of one part in 10^5 in the absolute value of K , which gives a completely insignificant change in the measured linewidth. The scattered light falls on the photocathode surface with nor-

mal incidence. The optics are shown in detail in Fig. 4.

The desirable characteristics of the photomultiplier are high gain, low noise, and optimum quantum efficiency at the laser frequency. Suitable photomultipliers are the RCA 7265; the ITT FW 130, or XP 1002, or the EMI 9558. The use of a photocathode with a small area, such as the ITT FW 130, reduces the level of dark current noise.

The simplest processing of the signal consists of the immediate a-c amplification of the voltage developed across the load resistor of the photomultiplier by a wide-band, linear-response audio amplifier. We use a Philbrick Nexus FSL-4 operational amplifier at a voltage gain of 84 which permits a flat frequency response from 5 to 100,000 hertz.

The spectrum of the photocurrent is obtained with an audiofrequency spectrum analyzer. The output signal of the spectrum analyzer is the Fourier transform of the voltage signal at the input. We use a Singer Panoramic LP-1a for Doppler broadenings of less than 2.5 kilohertz and a Singer Panoramic SP-3a for larger widths. Other equally acceptable spectrum analyzers would be the Hewlett-Packard 302a or the General Radio 1900a.

Two methods can be used to extract the signal from the very high shot noise of the photomultiplier tube. A very slow, spectrum-analyzer sweep speed, up to several hours, may be used in conjunction with a strip chart recorder having a very long time constant to measure the analyzer output. Although this method gives satisfactory results, we favor the use of a small digital signal averager. We use a fairly fast sweep speed of the spectrum analyzer, $\frac{1}{2}$ to 4 minutes, and repeatedly scan the output of the analyzer across the memory of the 400-channel digital signal averager, Nuclear-Chicago Model 24-2. In this way, averaging may be continued until inspection shows that the signal-to-noise ratio has been raised to an acceptable level. Repeated scanning of the spectrum in this manner also eliminates the effects of any long-term drift in the intensity of the laser output and mitigates the effects of any short-term variations. We obtain useful spectra in as short a time as 10 minutes with 20 sweeps; on the other hand, the averaging may be continued to give almost noiseless spectra (Fig. 5).

For general work, the half-width of the curve may be obtained by measuring the linewidth as displayed on an X-Y recorder; we estimate that the accuracy

obtainable by this method is better than 5 percent. For more accurate work the three parameters of the curve, the half-width at half-height, the intensity at zero frequency, and the intensity at "infinite" frequency may be obtained by a least-squares computer fit to the data, as presented by a digital printout of the signal averager memory. We believe that this method gives the half-width to better than 1 percent.

The laser and all the optical components are mounted on a 1-ton machinists' surface plate suspended from the ceiling by a modified Julius suspension (12). The cables from which the surface plate hang are attached to a thick plywood sheet that rests on a layer of inner tubes, which, in turn, are supported by a second plywood sheet hung directly from the ceiling. This procedure eliminates building sway and vibration.

We believe that, for the most accurate results, it is necessary to enclose the scattering cell in a thermostatically controlled environment. Viscosity effects alone represent a 5 percent increase in half-width for a rise of 2°C in temperature at 20°C, and this does not take into account possible broadening that may be introduced as a result of the establishment of convection currents in the solution. Except for measurements in the neighborhood of critical points, the degree of temperature stabilization required is not high; a variation of a few hundredths of a degree Celsius is tolerable. The temperature control is provided by a recirculating air stream heated with six GE quartz-line infrared lamps and controlled with a Hallikainen Thermotrol, model 1053a. We enclose the photomultiplier in the thermostatically controlled box also, despite the slight increase in the dark current noise.

To eliminate the effect of airborne dust, we find it necessary to baffle the airflow within the box. The beams are shielded from particles in the air by enclosing them in blackened tubes whenever they traverse an air space.

Experimental Technique and Results

The systems most extensively studied have been dispersions of polystyrene spheres in water (13-16). For rigid spheres the translational diffusion coefficient, D , can be computed a priori from the Stokes-Einstein relation

$$D = kT/3\pi\eta d \quad (19)$$

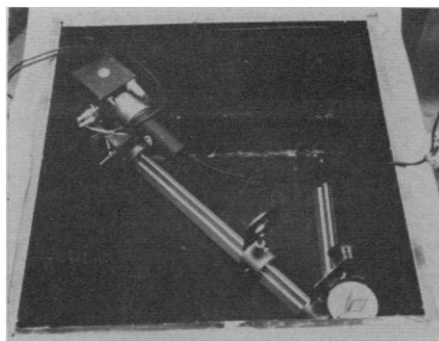


Fig. 4. Close-up view of the optics. In the foreground are the condensing lens, the scattering cell, and the aperture system defining the scattered beam. In the center of the optical rail is the collecting lens, and at the end of the rail is the shielded photomultiplier tube.

where k is Boltzmann's constant, T is absolute temperature, η is the viscosity of the medium, and d is the particle diameter. Using Eqs. 5, 18, and 19, we can compute the expected full-width at half-height, in radians per second, of the photocurrent power spectrum, $\Delta\omega_1$, which equals $2K^2D$

$$\Delta\omega_1 = \frac{8K_0^2 \sin^2(\theta/2) kT}{3\pi\eta d} \quad (20)$$

The experimentally observed linewidths and their angular dependence are in good agreement with Eq. 20. Dubin and co-workers (14) have extended the technique to bovine serum albumin and other large molecules. They find excellent agreement with diffusion coefficients measured by other techniques. Dunning's work in our laboratory on polystyrene spheres with diameters ranging from 854 to 2204 Å at temperatures from 23° to 38°C showed an average deviation from the predicted linewidths of only 6 percent.

The precision of a single measurement of the Doppler-broadened linewidth is extremely high, about 1 percent on a broadening of 1 kilohertz, which is itself a resolution of one part in 10^{11} . However, the accuracy in a series of experiments is often somewhat lower, especially if the optical alignment has been extensively changed between successive runs. This was particularly noticeable in the case of solutions of low scattering intensity and was attributed to the fact that a greater proportion of the light reached the detector, having been scattered from stationary surfaces such as the cell walls.

In experiments with reasonably concentrated solutions of polystyrene spheres (about 1 part of solids to

10,000 parts water) and reasonably large spheres (about 1000 Å), the reproducibility was excellent, better than 2 percent, indicating that the proportion of unbroadened light was insignificant. It should be noted that these results were obtained with an ordinary laboratory sample tube (12.7 by 1.3 cm) as the scattering cell and with only a single aperture to define the scattering path.

With smaller, more weakly scattering molecules, such as the MS-2 bacteriophage (17) with a diameter of around 400 Å, reproducibility was poorer; this we attribute to the decreased ratio of the light scattered from the solution to that scattered from stationary surfaces. These problems can be minimized by very careful definition of the scattered path by a series of apertures, by the use of a light trap to extinguish the transmitted beam, and by the use of sample cells of a very high optical quality.

Another method of reducing the light scattered from stationary surfaces has been suggested by Arrechi (15). This method consists of inserting the cell into the incident path at the Brewster angle, about 57°. We have also used cells with a multilayer dielectric coating on the cell walls to further reduce the scattering.

We examined the effect of multiple scattering by studying monodisperse solutions of 1000-Å polystyrene spheres, the ratio of spheres to water varying from 2:2500 to 2:100,000. The most concentrated solution scattered 75 percent of the incident light in a cell having a path length of 2.5 cm, and the most dilute solution represented the lowest scattering intensity that could be conveniently analyzed. These experiments were performed at a scattering angle of 45°. We were unable to detect any change in the linewidth between the most concentrated solution and one containing two parts of spheres in 50,000 parts of water, a twentyfold dilution range. In the most dilute solution, however, we did notice a decrease of about 10 percent in the linewidth, which we attribute to the increasing proportion of the spurious, unbroadened radiation being detected. The effect of the concentration on the half-width is, therefore, small.

Initial measurements on submicron particles, such as 1000-Å polystyrene spheres, indicated that the laser self-beat spectrometer was much less sensitive to stray scattering from large dust particles or other heterogeneous scattering

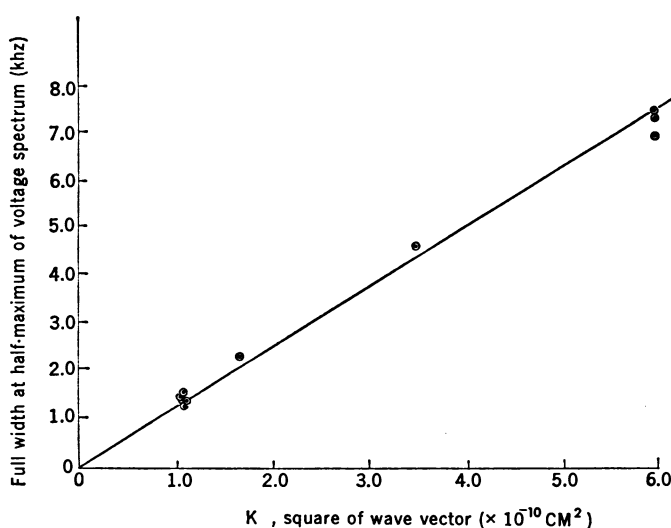
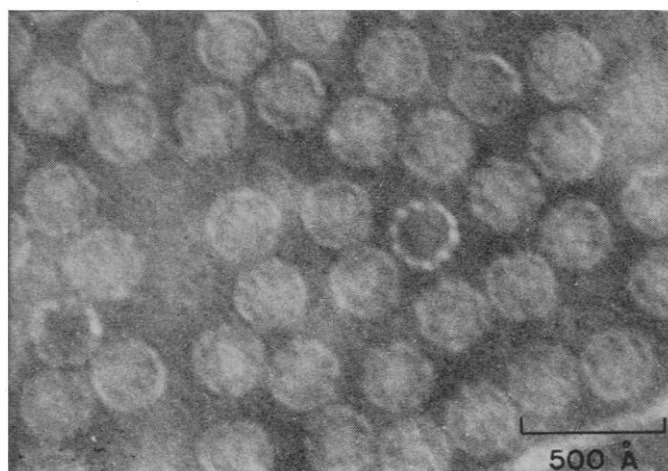
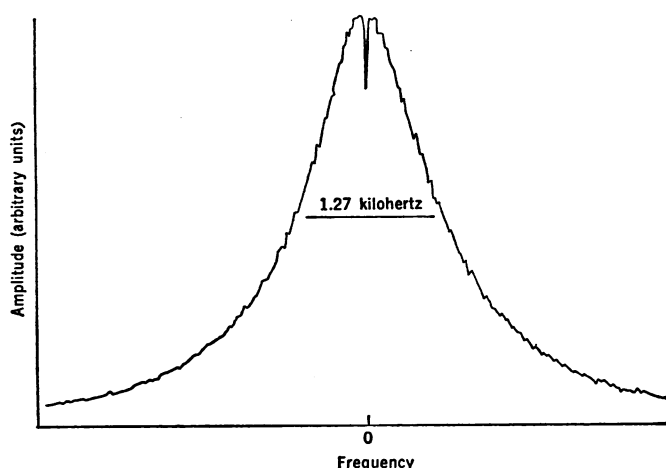


Fig. 5 (top left). Voltage spectrum of an aqueous solution of MS-2 bacteriophage at a scattering angle of 45° and a temperature of 23.5°C . The sharp dip in the center of the spectrum is an instrument characteristic at zero hertz (at the spectrum analyzer IF frequency). The spectrum was averaged for 224 sweeps of 4 minutes each.

Fig. 6 (top right). Electron micrograph of MS-2 bacteriophage.

Fig. 7 (left). Angular dependence of the half-width of the voltage spectrum of light scattered from an aqueous solution of MS-2 bacteriophage at 23.5°C .

centers than other means of measuring particle sizes—such as by angular dissymmetry. This is due to the fact that the latter relies entirely on the angular dependence of the intensity of the scattered light which is extremely sensitive to large particles that are, of course, very efficient scatterers. On the other hand, with the laser spectrometer, the particle size is estimated from the line-width of the scattered radiation; and since large particles have small diffusion coefficients, they give only small broadenings. Their only effect, therefore, is to introduce an abnormally intense signal around zero frequency. Usually this contribution to the spectrum may be eliminated by manually fitting the curve around zero frequency or by obtaining a least-squares computer fit to the tails of the curve. Nevertheless, it appears preferable to attempt to remove the majority of the large particles. We have used the conventional techniques of ultracentrifugation or filtration through Millipore filters.

When very weakly scattering solu-

tions are examined, contributions to the scattering from other types of fluctuations may become significant. Scattering from thermal phonons (Brillouin scattering) or from entropy fluctuations will each produce spectral components centered at zero frequency in a self-beat experiment. The relative importance of these other scattering processes can be assessed by performing the experiment on the pure solvent.

The spectrum obtained from a polydisperse system will, in general, differ from that of a monodisperse system. Dunning (18) has computed the effect of various simple types of polydispersity on the spectrum. He found that the changes are small and that laser beat frequency spectroscopy, at present, is not particularly useful for detecting or measuring polydispersity.

As an illustration of the self-beat technique, we present results obtained on a solution of MS-2 bacteriophage (17). An electron micrograph (Fig. 6) shows that the phage is spherical, with a diameter of approximately 250 Å. In

Fig. 5 we show the spectrum of light scattered from a aqueous solution of the phage (10 mg/liter). The spectrum is the voltage spectrum, which is proportional to the square root of the power spectrum of the photocurrent. The signal displayed is the heterodyne signal obtained by mixing the input signal with an internally generated intermediate frequency (IF) signal in the spectrum analyzer. Hence the spectrum appears as a symmetrical peak about the IF frequency, which corresponds, of course, to zero frequency of the input signal.

It can be shown by computer fitting that the curve illustrated is accurately described by the square root of the righthand side of Eq. 18. The diameter of the phage may be obtained directly from Eq. 20 in the form

$$\Delta\nu_{1/2} = \frac{32(3)^{1/2} n^2 k T \sin^2(\theta/2)}{3 \lambda_0^2 \eta d} \quad (21)$$

where $\Delta\nu_{1/2}$ is the full width, in hertz, at the half maximum of the displayed voltage spectrum, η is the viscosity, n is the refractive index of the fluid, and

λ_0 is the wavelength, in a vacuum, of the laser radiation. Equation 21 gives a value for the diameter of the phage in solution of 411 ± 10 angstroms compared to 250 angstroms obtained by electron microscopy of the dry phage. We believe that this deviation is real; that is, the phage has a somewhat greater diameter in solution than in the electron microscope. (The line widths that we obtain under similar conditions with dispersions of polystyrene spheres which are known to be of unchanging diameter in the electron microscope agree, within a few percent, with the diameter calculated from Eq. 21.)

In Fig. 7 we show the angular dependence of $\Delta\nu_{\frac{1}{2}}$ from our experiments on the bacteriophage. A linear relation between the half-width and $\sin^2(\theta/2)$ is found as predicted by Eq. 21.

We saw earlier that a contribution to the spectrum arising from the rotational decay of concentration fluctuations can only assume significance in the case of rod-shaped molecules for large scattering angles θ . This may be illustrated by the work of Dubin *et al.* (14) and Herbert *et al.* (19) on tobacco mosaic virus. This virus is a rod-shaped molecule about 150 Å in diameter and 3000 Å long. Dubin demonstrated that at large scattering angles the spectral distribution of the scattered light was not explicable on the basis of a single Lorentzian curve, corresponding to a single-decay mechanism for the dielectric constant fluctuation.

Herbert has shown that at scattering angles less than 90° the spectral distribution of the scattered light can be accurately described by a single Lorentzian, corresponding to translational diffusion. From the broadening of the

power spectrum at scattering angles less than 90° , he obtained a value for the translational diffusion coefficient of tobacco mosaic virus. The value obtained may then be used to calculate the contribution to the spectra from translational diffusion at scattering angles greater than 90° . This contribution may then be subtracted from the observed spectra, and the residue may be fitted to a second Lorentz curve to obtain the rotational diffusion coefficient.

Summary

We have seen that the spectral distribution of light scattered from spherical molecules and from rod-shaped molecules may be analyzed in terms of the translational and rotational diffusion coefficients of these molecules. These coefficients may, in turn, be related to the size and conformation of the molecules.

In addition, laser beat frequency spectroscopy can be used to analyze the spectrum of light scattered from pure liquids near their critical points and solutions near their critical mixing points. These phenomena are fully discussed by Alpert, Yeh, and Cummins (20), Ford and Benedek (10), Lastovka and Benedek (11), Mountain (21), Chu (22), and in the book by Fabelinskii (23).

A theoretical calculation of the spectral distribution expected of the light scattered from fluctuations about equilibrium in a chemically reactive solution has been made by Blum and Salsburg (24). Laser spectroscopy may, therefore, find application in the measurement of the relaxation rates of fast reactions. In our laboratory we are using laser self-beat spectroscopy to investigate the con-

formational changes that occur in biomolecules under the influence of environmental changes such as changes in pH, temperature, and dielectric constant of the solvent.

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