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OBSERVATION OF THE SPECTRUM OF LIGHT SCATTERED BY SOLUTIONS OF BIOLOGICAL MACROMOLECULES*

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Using a laser light source, in conjunction with an optical "self-beat" spectrometer[1-3] whose resolving power is of the order of 10^{14}, we have observed the spectral distribution of light scattered by dilute solutions of several natural and synthetic macromolecules, namely polystyrene latex spheres, bovine serum albumin, ovalbumin, lysozyme, tobacco mosaic virus, and deoxyribonucleic acid. From the spectrum of the scattered light, we have been able to determine the diffusion constants (D) of these macromolecules with a precision of typically 3 per cent.

Theory of the Scattering.—The theory that describes the scattering of light has been clearly presented in a number of publications.[4-14] There are two rather well-defined theoretical approaches to the case of light scattering by solutions of macromolecules. The first is a continuum theory which applies when the orientation of the molecules does not affect the scattering; otherwise, the second approach, a molecular theory of the scattering, must be used. We present first the results of the continuum theory, in which the scattering results from fluctuations in the dielectric constant (ϵ) about its average value. More exactly, by Fourier-analyzing the fluctuations in terms of plane waves, it can be shown[6] that the light scattered at an angle Θ from the incident beam is the result of a Bragg reflection[7, 8] of the incident beam by a fluctuation in dielectric constant $[\delta\epsilon(\lambda_f,t)]$ whose wavelength (λ_f) satisfies the Bragg law

$$(\lambda_0/n) = 2\lambda_f \sin(\Theta/2), \tag{1}$$

where λ_0 is the wavelength of the incident light in vacuum and n is the index of refraction of the scattering medium. The amplitude of the scattered electric field is directly proportional to the amplitude of the fluctuation $[\delta\epsilon(\lambda_f,t)]$ which produced it, while the time dependence of the scattered field[9] has the form of the incident field modulated by the time dependence of the fluctuation in dielectric constant. That is, the scattered field $[E_S(\mathbf{R},t)]$ at a point $\mathbf{R}$ and time t is proportional to $e^{2\pi i\nu_0 t}\delta\epsilon(\lambda_f,t)$, where ν_0 is the frequency of the incident field. Now, $\delta\epsilon$ in turn is produced to a high degree of approximation by the corresponding fluctuation in the concentration $[\delta C(\lambda_f,t)]$ through the relation

$$\delta\epsilon(\lambda_f,t) = (\partial\epsilon/\partial C)\delta C(\lambda_f,t). \tag{2}$$

The *intensity* of the scattered light is proportional to $\langle|\delta\epsilon|^2\rangle$, and once $(\partial\epsilon/\partial C)$ is measured, the light intensity is a known constant multiple of $\langle|\delta C|^2\rangle$. But according to the theory of fluctuations,[5, 10] this latter quantity is simply proportional to $M\langle C\rangle$ where M is the molecular weight and $\langle C\rangle$ is the average concentration. Thus the intensity of the scattered light is used to measure the molecular weight of the solute.[4, 5]

In the present experiment the *spectrum* of the scattered light is studied. Debye[8] has calculated the spectrum under the presumption that the concentration fluctua-

tions obey the diffusion equation $\{\partial/\partial t[\delta C(\mathbf{r},t)] = D\nabla^2[\delta C(\mathbf{r},t)]\}$. Solving this equation for a fluctuation in concentration having wavelength λ_f and which appears at $t = 0$, we see that this fluctuation will decay exponentially to zero at a rate (Γ_D) given by

$$\Gamma_D = D(2\pi/\lambda_f)^2 = DK^2, \tag{3}$$

where D is the diffusion constant and $K \equiv (2\pi/\lambda_f)$, the wave vector of the scattering fluctuation. If we employ the Bragg reflection condition [eq. (1)], we may express the decay rate (Γ_D) as a function of the scattering angle (Θ) by

$$\Gamma_D = D\left[\frac{4\pi}{(\lambda_0/n)} \sin(\Theta/2)\right]^2. \tag{4}$$

The exponential decay implies a Lorentzian spectrum. To be precise, if $S(\nu,\Gamma_D)$ is the power spectrum of the scattered light, then [9, 11]

$$S(\nu,\Gamma_D) \propto \left[\frac{(\Gamma_D/2\pi)}{(\nu - \nu_0)^2 + (\Gamma_D/2\pi)^2}\right]. \tag{5}$$

The half width at half height of this spectrum is $\Gamma_D/2\pi$. Thus measurements of the half width, the scattering angle, and the index of refraction permit the determination of the diffusion constant of the macromolecule.

Since D for macromolecules ranges[5] from about 10^{-8} cm^2/sec to about 10^{-6} cm^2/sec, we expect that the half widths of the spectra observed in the backward direction [$\Theta = 180°$] will fall in the range 100 Hz $< (\Gamma_D/2\pi) <$ 10 kHz. The extreme narrowness of these lines necessitates the use of a laser light source and the "self-beat" spectrometer described below.

Before proceeding, we must discuss the molecular theory for the spectrum of the scattered light. Such a theory has been presented by Pecora[12–14] in which the spectral distribution given above arises from the isotropic diffusional motion of the center of mass of each of the molecules. In addition to this motion the molecules undergo rotational motion relative to their center of mass. This rotation modulates the scattered light and thus affects the angular dependence and the shape of the spectrum.[15] We can therefore expect that for nonspherical molecules with dimension comparable with the wavelength of light, the spectrum of the scattered light will contain information regarding both the rotational and translational diffusion of the macromolecules.

Experimental Methods.—If we are to resolve the narrow lines whose widths have been predicted above, the resolving power ($\mathcal{R} = \nu_0/\Delta\nu$) of the spectrometer must fall into the range 5×10^{11} to 5×10^{13}. Grating spectrometers have $\mathcal{R} < 8 \times 10^5$, while the best spherical Fabry-Perot etalons have $\mathcal{R} < 5 \times 10^7$. Thus, the resolution required in the present experiment is a factor of 10^4 to 10^6 higher than that available with the best conventional spectroscopic methods. The required resolution can be achieved using the newly developed techniques of optical heterodyne and "self-beat" spectroscopy[1, 2, 3, 16, 17] in conjunction with laser light sources. These new techniques have already been used to study the spectrum of light scattered by concentration fluctuations in suspensions of polystyrene molecules,[16] and in binary mixtures near their critical mixing temperature.[18–20] In addition, they have been successfully employed in recent experiments to study the spectrum of light which is scattered by pure fluids near their critical point[2, 3, 21] and the spectrum of light scattered by entropy fluctuations in a normal fluid.[17]

The essential feature of these techniques is to transfer the spectral information initially centered at the optical frequency to a much lower frequency where conventional electrical filters may

conveniently be used to analyze the spectrum. This transfer is accomplished in the "self-beat" spectrometer[2, 3] by allowing the scattered light to fall on the surface of a photomultiplier tube. The output current of this device is proportional to the *square* of the incident electric field. As a result the photocurrent contains beat notes between each of the spectral components of the light falling on the photomultiplier. The spectral information which was initially present in the scattered electric field now appears in a slightly modified form in the spectrum of the photocurrent which is centered at zero frequency. To be quite specific, if the power spectrum of the light is Lorentzian, centered at $\nu = \nu_0$, with half width $(\Gamma_D/2\pi)$, the "self-beat" power spectrum of the photocurrent is also Lorentzian, but its center frequency is at $\nu = 0$ and its half width at half height[2, 3] is $2(\Gamma_D/2\pi)$. In addition to this, the spectrum of the photocurrent contains a frequency-independent shot noise part proportional to the magnitude of the d-c photocurrent. The spectrum of the photocurrent is measured using a conventional audio spectrum analyzer. This analyzer is a narrow band tuned filter whose center frequency may be swept. The analyzer output is a d-c signal proportional to the *amplitude* of the frequency components of the photocurrent which fall within the passband of the analyzer. By definition the spectrum of the photocurrent is the square of these amplitudes. Thus in order to obtain the spectrum directly, the analyzer output is passed through an analogue squarer prior to recording.

The block diagram is given in Figure 1. The incident light is provided by a Spectra-Physics model 125 helium-neon laser ($\lambda_0 = 6328$ Å). Parallel rays of light scattered at an angle Θ are collected by a spherical lens and focused onto the surface of an RCA 7265 photomultiplier tube. The scattering angle Θ is influenced by refraction of the light as it passes from the scattering medium into the air. Thus the index of refraction of the solution must be measured to determine Θ, as well as K. The range of angles $\Delta\Theta$ which are accepted is determined by the size of a pinhole placed before the phototube. The phototube output was analyzed with either a General Radio 1900A wave analyzer (for lines up to 5 kHz wide) or with a Hewlett Packard 310A spectrum analyzer (for lines wider than 5 kHz). The time needed to record a typical trace was about $1^1/_2$ hours. The temperature at which our data was taken was that of the room.

Experimental Results.—Using the experimental methods described above, we have studied the spectrum of light scattered by aqueous solutions of the following macro-

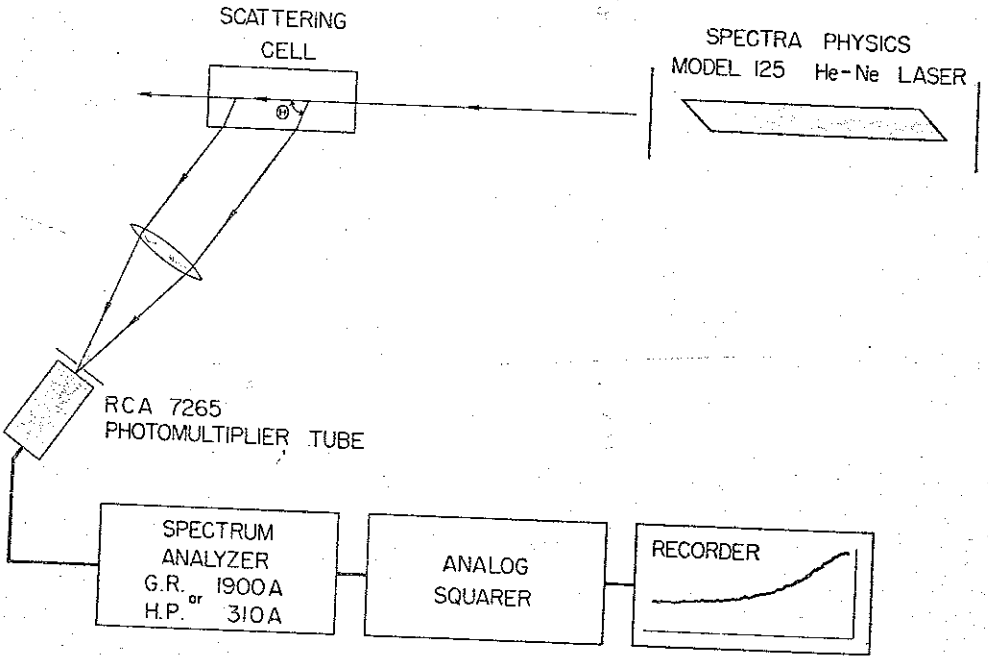

FIG. 1.—The experimental apparatus.

molecules: polystyrene latex spheres, bovine serum albumin, ovalbumin, lysozyme, tobacco mosaic virus, and deoxyribonucleic acid.[22] Our results are summarized in Table 1, which lists the concentration of the macromolecule in mg/cc, the pH, the salt content of the solution, and our determination of the translational diffusion constant (D). For comparison we list values of D determined by more conventional methods.[5] Since the concentrations used in the other measurements were generally different from ours, we list in the comparison column their concentrations. To facilitate comparison we have adjusted all the data on D to apply to 25°C. This was done by using the Stokes-Einstein relation [eq. (6) below] and the known temperature dependence of the viscosity of water. In no case did this correction exceed 5 per cent.

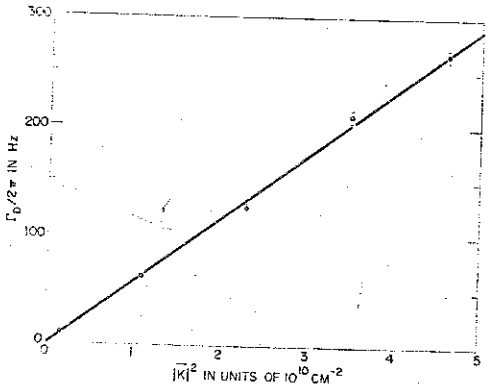

FIG. 2.—$(\Gamma_D/2\pi)$ versus K^2 for an aqueous solution of polystyrene latex spheres of radius 630 Å.

Polystyrene latex spheres: We studied in considerable detail the spectrum of light scattered by three aqueous solutions of polystyrene latex spheres of known radius ($r = 440 \pm 40$ Å, 630 ± 30 Å, and 1830 ± 30 Å). This system is most useful as a test of the theory and the experimental method since the scattering from each molecule is independent of the molecular orientation and because the diffusion constant (D) can be predicted *a priori* from the Stokes-Einstein relation:[23]

$$D = \frac{k_B T}{6\pi\eta r}, \tag{6}$$

where k_B is Boltzmann's constant, T is the temperature in °K, η is the solute viscosity, and r is the macromolecular radius. The spectrum of the scattered light was studied as a function of angle for scattering angles between 0 and 110° for the 630-Å spheres, and for a single angle for the 440-Å spheres [17°] and the 1830-Å spheres [90°]. The line shapes were accurately Lorentzian. In Figure 2, $(\Gamma_D/2\pi)$ versus K^2 is plotted for the 630-Å spheres. The line width varied from 10 Hz to 270 Hz in accordance with the K^2 dependence predicted in equation (3). The value of D deduced from the slope of this graph is in excellent agreement with the value determined from the Stokes-Einstein relation (Table 1). Similarly, the values of D deduced for the 440-Å and 1830-Å spheres from the single measurements agree with the theoretical values. The uncertainty in the theoretical values is determined solely by the uncertainty in the knowledge of the sphere radii. We may conclude that the concentration fluctuations in suspensions of these polystyrene spheres accurately obey the diffusion equation even over dimensions comparable to the wavelength of light.

Bovine serum albumin (BSA): The spectrum of light scattered by two solutions of this protein (characteristic dimension 100 Å) was studied as a function of scattering angle from 17 to 151°. The solvent in the first solution was water with negligible salt content, while the second solvent was a 0.5 M KCl solution. Figure 3 shows

TABLE

DIFFUSION CONSTANTS OF SEVERAL

Sample	Concentration (mg/cc)	pH	
Bovine serum albumin	30	6.91	
Ovalbumin	30	6.80	
Lysozyme	50	6.80	
Tobacco mosaic virus	60	5.60	
DNA (calf thymus)	0.1	7.20	
	0.5	7.00	
Polystyrene latex spheres $r = 440$ Å $\pm$ 40 Å	0.03	7.40	
Polystyrene latex spheres $r = 630$ Å $\pm$ 30 Å	0.03	7.40	
Polystyrene latex spheres $r = 1830$ Å $\pm$ 30 Å	0.03	7.40	

[a] The probable accuracy of the values of D cited in the literature is only about 5% (ref. 5, p. 360).
[b] No comparison value is available.
[c] Wagner, M. L., and H. A. Scheraga, *J. Phys. Chem.*, **60**, 1066 (1956).
[d] Lamm, O., and A. Polson, *Biochem. J.*, **30**, 528 (1936).

the spectrum of the photocurrent for light scattered at $\Theta = 151°$ for the 0.5 M KCl solution. This trace took 90 minutes to record. The frequency-independent spectral power density which is clear at 300 kHz represents the shot noise contribution. The spectrum above this shot noise level was fitted with a Lorentzian of half width at half height $(2\Gamma_D/2\pi) = 13.9$ kHz. The form of this Lorentzian fit is shown by the open circles, which indicates that the spectrum is accurately Lorentzian, as were all the spectra observed in the study of BSA. The display of $(\Gamma_D/2\pi)$ versus K^2 is given in Figure 4 and shows that the line width varies accurately with K^2 for both solutions as predicted by equation (3). However, the values of D given in Table 1 are markedly different in the two cases. It is interesting to observe that the diffusion constant in distilled water is over 50 per cent larger than in the 0.5 M KCl solution, even though the solvent viscosities as measured macroscopically are nearly identical for the two solutions. This suggests that the effective size of the serum albumin molecules is substantially smaller in the absence of salts. It was also observed that if the distilled water solution was allowed to stand for a period of 24–48 hours, the diffusion constant increased. The data presented here were all obtained within two hours after sample preparation.

Our value of $[6.7 \pm 0.1] \times 11^{-7}$ cm^2/sec for D in the salt solution is in good agreement with the result obtained by conventional means. It might be mentioned that the precision of the present determination is quite high ($\pm 1.5\%$), and the relative speed with which the measurement can be made has made it possible for us to detect the increase of D with time in the salt-free solution. While the concentration of the solution was several times higher than that used in the conventional method, we could reduce the concentration needed to about 1 mg/cc if we confined

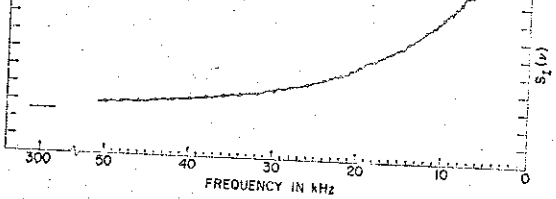

FIG. 3.—The spectrum of the photocurrent for light scattered at $\Theta = 151°$ by bovine serum albumin in a 0.5 M KCl solution. The open circles represent a Lorentzian line of 13.9 kHz half width.

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NATURAL AND SYNTHETIC MACROMOLECULES

Salt content	Present method D (in units of 10^{-7} cm²/sec)	Comparison[a] D (in units of 10^{-7} cm²/sec)	Comparison[a] Concentration (mg/cc)
—	10.2 ± 0.2	[b]	—
0.5 M KCl	6.7 ± 0.1	6.7[c]	12.5
0.5 M KCl	7.1 ± 0.2	8.3[d]	14
—	11.5 ± 0.3	11.6[e]	8
0.01 M Sodium phosphate buffer	0.40 ± 0.02	0.3[f]	2.2
0.15 M NaCl	0.2 ± 0.1	0.13[g]	0.5
0.015 M Trisodium citrate			
—	0.59 ± 0.02	0.56 ± 0.06[h]	—
—	0.368 ± 0.006	0.38 ± 0.02[h]	—
—	0.134 ± 0.004	0.134 ± 0.002[h]	—

[a] Colvin, J. R., *Can. J. Chem.*, 30, 831 (1952).
[f] Schachman, H. K., *J. Am. Chem. Soc.*, 73, 4808 (1951).
[g] Ref. 5, p. 361.
[h] Computed directly from equation (6).

our measurements to small scattering angles where the spectral power per unit bandwidth is high.

Ovalbumin and lysozyme: These macromolecules have characteristic sizes (100 and 50 Å, respectively), small compared with the wavelength of light. The spectrum of light scattered by an aqueous solution of lysozyme and a 0.5 M KCl solution of ovalbumin was studied for $\Theta = 90°$ in each case. The spectra observed were accurately Lorentzian and the values of D which were deduced are listed in Table 1 along with the comparisons with previous determinations.

Tobacco mosaic virus (TMV): Since TMV is rod-shaped with dimensions about 3000 Å × 150 Å, one would not expect the continuum theory presented above to apply. This arises because the molecule has dimension comparable to the wavelength of light, and its rodlike shape probably leads to anisotropic diffusion of the center of mass of each molecule. The spectrum of the scattered light was studied for several scattering angles between 0 and 90°, and the spectra were not generally Lorentzian, except for 90° scattering. The observed spectra could generally be fit with a Lorentzian curve if a width $2(\Gamma/2\pi)$ was determined by fitting the data in the "tails" of the trace, i.e. for $\nu > 2(\Gamma/2\pi)$, but this fit became poor as ν approached zero. In fact, at $\nu = 0$, the Lorentzian which fit well in the "tails" of the trace was substantially too low [typically a factor of 2 or more]. These half widths, however, did vary with scattering angle approximately as $\sin^2(\Theta/2)$ for the range of angles studied. In Table 1 we give the value of D obtained from Lorentzian-shaped traces taken at a scattering angle of 90°. The results in the present case clearly point to the importance of a molecular theory of scattering[12–14] which includes the effects of molecular reorientation and anisotropic diffusion on the spectra.

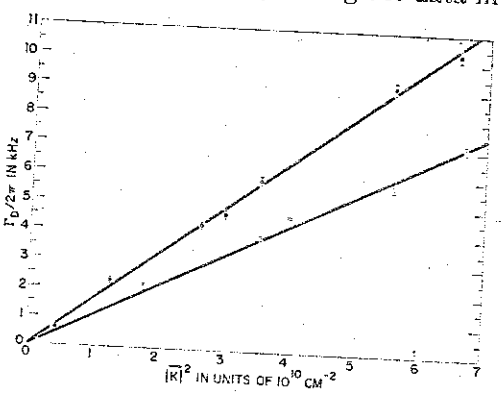

FIG. 4.—$(\Gamma_D/2\pi)$ versus K^2 for light scattered by solutions of bovine serum albumin. The solid circles refer to a 30 mg/cc concentration of bovine serum albumin in distilled water, while the open circles refer to the same concentration in 0.5 M KCl.

Deoxyribonucleic acid (calf thymus DNA): The preparation of DNA used contained molecules ranging in molecular weight between 4,000,000 and 6,000,000. The DNA molecule has a somewhat flexible form and has dimension comparable to the wavelength of light; as a result, we have here another case in which Lorentzian lines cannot be expected. We recorded the "self-beat" spectrum of the light scattered at $\Theta = 90°$. Since it was found that this trace could not be fit to a single Lorentzian, the value of D which is listed in Table 1 was determined by equating $2(\Gamma_D/2\pi)$ to the half width at half height of the trace. The uncertainty listed in Table 1 represents the range of widths one obtains by choosing a Lorentzian which fits in the "tails" but not in the center, or vice versa. Again, in the case of this molecule a more detailed molecular theory is required for spectral analysis.

Conclusions.—The results presented above demonstrate that for molecules whose characteristic dimensions are small compared to the wavelength of light, the spectrum of the scattered light provides reliable information on the translational diffusional motion of the molecule. The diffusion equation that describes the space and time dependence of the concentration fluctuation is shown to be accurately obeyed even over dimensions comparable to the wavelength of light. On the other hand, the spectrum of the light scattered by molecules whose dimensions are comparable with the wavelength of light cannot be completely described on the basis of isotropic translational diffusion. We expect that the analysis of later data will yield information on both rotational and anisotropic translational diffusion of these large macromolecules.

The method of spectral analysis presented here, employing a laser light source and a "self-beat" spectrometer, has the following attractive features: (*a*) The method is accurate. The data in Table 1 show that D can be determined with a precision of 3 per cent or better. (*b*) The method uses only spontaneous concentration fluctuations—no macroscopic concentration gradient need be established. (*c*) The concentration required to obtain useful spectra is quite low (about 1 mg/cc) if one studies the spectra for small scattering angles (Θ about 30° or less). (*d*) The "self-beat" spectrometer does not detect any uniform motion of the solution over the region from which the light is collected. As a result, to first order, convection does not affect the measurement of D. Thus in our measurements, it was not necessary to establish a very uniform temperature throughout the sample. All data were taken at room temperature without temperature controls. (*e*) The method is fast. It enables a determination of D within one hour or so after sample preparation. We believe that this technique for the study of macromolecular diffusion can be of considerable use in the observation and interpretation of structural changes in biological macromolecules.

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