Study of the Chemical Denaturation of Lysozyme by Optical Mixing Spectroscopy†

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ABSTRACT: We have applied the techniques of optical mixing spectroscopy to measure the translational diffusion coefficient (D) of lysozyme in buffered guanidine hydrochloride (Gdn·HCl) solutions. At pH 4.2 and 1% protein concentration, $D_{20,w}$ was determined for 31 values of the concentration of Gdn·HCl between 0 and 6.4 M. The diffusion coefficient $D_{20,w}$ remained essentially constant at $(10.6 \pm 0.1) \times 10^{-7}$ cm²/sec between 0 and 2 M Gdn·HCl concentration, and then decreased smoothly to $(7.3 \pm 0.1) \times 10^{-7}$ cm²/sec at 5 M Gdn·HCl, where it plateaued. These values of D in conjunction with other hydrodynamic parameters indicate that lysozyme retains a compact configuration upon denaturation. The spectral shape of the light scattered by lysozyme did not permit us to distinguish between different models of denaturation because the diffusion coefficient of native lysozyme is only 45% greater than that of the denatured protein. Assuming a model in which lysozyme can exist in only two states (native and denatured), we calculate the fraction of each state present at different concentrations of the denaturant. The transition region we observed in these studies has approximately the same width and occurs at about the same Gdn·HCl concentration as the transitions observed by optical rotation and specific viscosity. This indicates that all these techniques probe the same transition in lysozyme. Upon treatment of the protein at the highest Gdn·HCl concentration with 30 mM dithiothreitol the diffusion coefficient was lowered to $(5.7 \pm 0.2) \times 10^{-7}$ cm²/sec.

The determination of the translational diffusion coefficient (D) has been employed in the past predominantly in connection with the measurement of the molecular weight of large molecules by the technique of sedimentation–diffusion. Because of the difficulties in obtaining D by classical methods, this parameter has not been fully explored as a measure of molecular conformation and conformational alterations. The major problems presented by classical methods occur because of the necessity of very high temporal and spatial uniformity of temperature during rather long experimental runs. This requirement is eliminated by the recently developed techniques of optical mixing spectroscopy (Dubin *et al.*, 1967; Clark *et al.*, 1970; Dubin, 1970, 1972), which, additionally, shorten the time required for a determination of D to about 1 hr or less. In the present work, we have employed a "self-beating" spectrometer to determine the diffusion coefficient of lysozyme at 1% concentration for 31 values of the concentration of the denaturant, Gdn·HCl,[1] between 0 and 6.4 M. We have obtained typically 2% accuracy in experimental runs of 1–3 hr. Ultra-high-temperature stability was unnecessary since the self-beating spectrometer is insensitive to convection currents if the liquid flow is uniform across the illuminated volume. These two properties of the technique—rapid determination of D and insensitivity to small temperature gradients—render the method ideal for the present study.

The original motivation of these experiments was to determine whether optical mixing spectroscopy could distinguish between different models of the denaturation process. For the case of lysozyme it was not found possible to render such a distinction.

Theory

The theory of the scattering of light by concentration fluctuations and the method of determining D from the spectrum of this light by optical mixing spectroscopy have been described elsewhere (Pecora, 1964; Dubin *et al.*, 1967; Benedek, 1969; Dubin, 1970, 1972; Clark *et al.*, 1970; Rimai *et al.*, 1970; Foord *et al.*, 1970; Cummins and Swinney, 1970). We shall simply describe the principles here and quote the necessary results.

Light incident upon a solution of macromolecules will be scattered by fluctuations in the local polarizability of the medium. For macromolecular solutions, such fluctuations are due almost entirely to fluctuations in solute concentration. In particular, light of wave vector $\mathbf{k}$ will be scattered by a fluctuation in polarizability of wave vector $\mathbf{K}$ to produce scattered light of wave vector $\mathbf{k}_s$ if the condition $\mathbf{K} = \mathbf{k}_s - \mathbf{k}$ is satisfied. If the concentration fluctuations obey the diffusion equation, then the power spectrum of the scattered light is given as

$$S(\nu) \propto \frac{(\Gamma/2\pi)}{(\nu - \nu_0)^2 + (\Gamma/2\pi)^2} \tag{1}$$

where ν_0 is the frequency of the incident light and Γ is the decay rate of the concentration fluctuation which produced the scattering. We may write

$$\Gamma = DK^2 = D\left[\frac{4\pi n}{\lambda_0} \sin(\theta/2)\right]^2 \tag{2}$$

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[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

where λ_0 is the wavelength *in vacuo* of the incident light, n is the solution index of refraction, and θ is the angle between the incident and scattered light beams. The value of D is of the order of 10^{-6} cm²/sec for small enzymes, and thus the spectral half-width at half-maximum ($\Gamma/2\pi$) is typically only several kilohertz, even in the case of back-scattering ($\theta \gtrsim 180°$). To resolve such extraordinarily narrow spectral lines a laser light source and the techniques of optical mixing spectroscopy must be applied. In the case of the "self-beating" type spectrometer employed in the present experiments we measure the power spectrum of the photocurrent produced when the scattered light is detected by a photomultiplier tube. This spectrum is centered at zero frequency instead of at the frequency of the incident light and is given as

$$S_i(\nu) \propto \frac{(2\Gamma/2\pi)}{\nu^2 + (2\Gamma/2\pi)^2} \tag{3}$$

This spectrum is readily resolved by a conventional audio-frequency spectrum analyzer.

Materials and Methods

A twice-crystallized salt-free lysozyme preparation was employed in these studies (Worthington Biochemical Corp., lot No. 9FM). A 0.1 M sodium acetate–acetic acid buffer was used at pH 4.2 ± 0.1 at which value lysozyme is known to be monomeric (Sophianopoulos and van Holde, 1964; Bruzzesi *et al.*, 1965).

The Gdn·HCl used to denature the lysozyme was obtained from Mann Research Laboratories (New York). A single supply (lot no. U-2491) was employed for all experiments. Gdn·HCl concentrations were determined refractometrically using a Bausch and Lomb Model 3L Abbé refractometer and the data of Kielley and Harrington (1960). These data were corrected for the slight contribution to the refractive index of the buffer ions employed in the present study. Despite its grade of Ultra Pure, the Mann Gdn·HCl was found to contain a small amount of particulate contamination which could be seen clearly as a residue on a Millipore filter. It was found that clean lysozyme solutions were most readily obtained by prefiltering the buffered Gdn·HCl solutions *before* the addition of the lysozyme. Two filtrations of the solution through a 50-mμ cellulose ester Millipore filter removed all trace of the residue. One per cent (w/v) lysozyme solutions were then prepared and, after the scattering cell was cleaned as described below, the solutions were filtered into the cell through a 0.22 μ Millipore filter. The *solution* index of refraction, required both for the determination of K (eq 2) and for correcting the measured scattering angle to the actual scattering angle (Dubin, 1970) *via* Snell's law, was determined again using the Bausch and Lomb Model 3L Abbé refractometer. The scattering angle was determined to an accuracy (Dubin, 1970) of 20 min and was approximately 60° in all experiments.

Standard Teflon-stoppered spectrophotometric cuvettes were employed as scattering cells and were painstakingly cleaned and filled. First cleaned in chromic acid, the cells were rinsed in distilled water and ultrasonically cleaned in glacial acetic acid for 15 min. Four liters (approximately 500 cell volumes) of distilled water were then passed through the cell in a closed system under pressure. This water was filtered in-line through a 0.22 μ Millipore filter. To ensure a high flow rate (approximately one scattering cell volume per second) large size (14-cm diameter) filters were employed for this flushing procedure. Without opening the cell to air, filtered dry nitrogen was admitted to displace the remaining water and dry the cell. This cleaning procedure rendered the scattering cells sufficiently clean that, when filled with water, only the very weak Brillouin scattering (Fabelinskii, 1957) and anisotropy scattering (Fabelinskii, 1957) from water itself were visible. All particulate contamination was removed. With the cell prepared in this fashion, the 1% lysozyme solution, prefiltered several times through a 0.22 μ Millipore filter, was then admitted to the scattering cell. Even with this elaborate procedure, only about one cell filling in three was acceptable. A successful filling of the cell was one in which no angular dissymmetry could be detected in the scattered light and no particulate contamination could be seen in the unfocused laser beam upon observation through a 20× microscope. Before a spectral run was begun, the incident beam had to appear completely diffuse under these conditions. If these criteria were not met, the sample was not used and the above procedure was repeated.

All experiments were performed at room temperature without temperature controls. However, the temperature in the scattering cell was stable to better than 0.2° during the course of an experimental run. All data were obtained between 23 and 25° and corrected to standard conditions. Experimental runs were begun within 2 hr of sample preparation and typically lasted 1–3 hr, although some runs as long as ten hours were performed.

Solvent kinetic viscosities were determined using a Cannon-Manning semimicro capillary viscometer. The solvent viscosity, η, was in turn obtained using the Gdn·HCl density data of Kielley and Harrington (1960), correcting for the very slight increase in density due to the presence of the buffer ions. Our results at 25° were uniformly about 0.02 cP higher than those of Kawahara and Tanford (1966), the difference being due to the presence of the buffer ions employed in the present work. It should be noted that the increase in solution viscosity due to the guanidine hydrochloride is quite substantial; a 6 M Gdn·HCl solution, for example, has a viscosity 62% greater than water alone.

The self-beating spectrometer has been described extensively elsewhere (Dubin *et al.*, 1967; Clark *et al.*, 1970; Dubin, 1970, 1972) and will not be considered further here. It should be noted, however, that the fact that a 1% lysozyme solution is a rather weak scatterer required great stability of the present experimental apparatus (Dubin, 1970). The intensity of the laser radiation (50 mW at 6328 Å from a Spectra-Physics No. 125 He-Ne laser) was servo controlled to better than 0.05% and the output rendered noise free by use of its radiofrequency quieting. The system response of the wave analyzers (Hewlett-Packard 310-A and General Radio 1900A) was calibrated to better than 0.1% over their entire ranges. Stability of the phototube–wave analyzer combination was measured to be better than 0.1% for periods exceeding 12 hr. The spectrometer employed in these experiments (Dubin, 1970, 1972) allowed us to determine $D_{20,w}$ for lysozyme with a 1% accuracy and, in addition, even allowed us to determine the diffusion coefficient of guanidine hydrochloride as described in the next section.

It is noteworthy that since the initiation of these studies, two important advances have taken place in the equipment available for assembling optical mixing spectrometers. First, lasers with optical power outputs one to two orders of magnitude higher than that obtained from the laser employed here are now commercially produced (for example, Coherent Radiation Laboratories Model 52 series). Such lasers allow

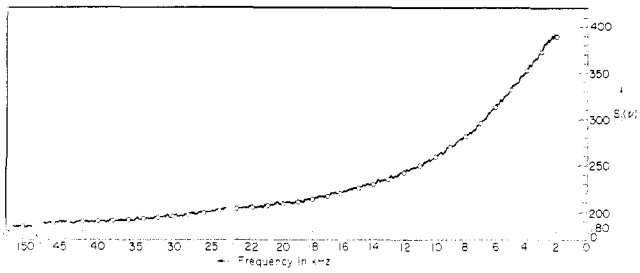

FIGURE 1: Self-beat spectrum of the light scattered at $\theta = 61.2°$ by a 1% solution of native lysozyme ([Gdn·HCl] = 0) at pH = 4.2 and $T = 24.2°$. The solid line is the experimental data, and the open circles represent the best Lorentzian fit (see eq 3 in text) to the data. The half-width of this fit is $(2\Gamma/2\pi) = 6872$ Hz, from which $D_{20,w}$ was determined to be 10.7×10^{-7} cm²/sec.

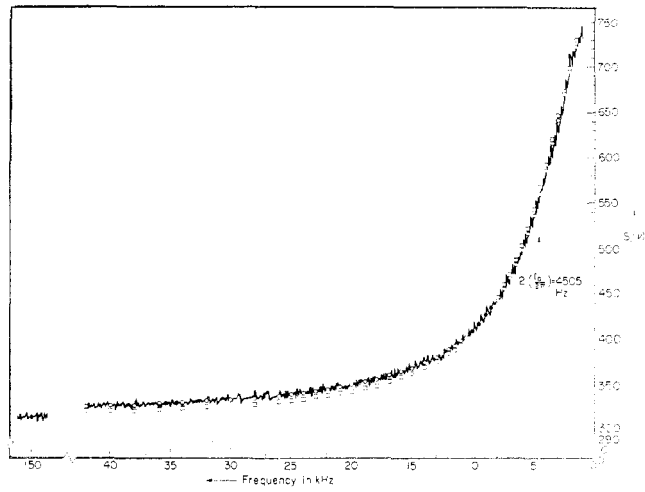

FIGURE 2: Self-beat spectrum of the light scattered at $\theta = 62.9°$ by a 1% solution of lysozyme in the presence of 4.37 M Gdn·HCl at pH 4.2 and $T = 23.7°$. The solid line is the experimental data, and the open squares represent the best *single* Lorentzian fit (see eq 3 in text) to the data. The half-width of this fit is 5077 Hz. The open circles show the best *two* Lorentzian fit (see eq 5 in the text) with $(2\Gamma_L/2\pi) = 4505$ Hz and $(\Gamma_L + \Gamma_G)/2\pi = 22.39$ kHz. From these widths we determined $D_{20,w}$ for lysozyme to be 8.02×10^{-7} cm²/sec and $D_{20,w}$ for guanidine hydrochloride (see text) to be 54×10^{-7} cm²/sec.

experiments with the same signal-to-noise ratio as obtained in this study to performed in considerably shorter periods of time (Dubin, 1970, 1972). In addition, multichannel wave analyzers (for example, Federal Scientific's Ubiquitous series or the Saicor spectrum analyzer) and digital autocorrelators[2] (Foord *et al.*, 1970), such as the Saicor correlation analyzer have made possible another very substantial reduction in the time required to obtain satisfactory results. At present, state-of-the-art technique can yield enzyme diffusion coefficients in a time of the order of 1 min. In fact, the data we present at 6.4 M Gdn·HCl and the data on the effect of dithiothreitol on lysozyme were obtained using a Saicor 400-channel real-time spectrum analyzer (Model SAI-52A) in a time period of about 5 min.

Results

In Figure 1, the solid line is the self-beat spectrum of the light scattered at $\theta = 61.2°$ by a 1% lysozyme solution in the absence of guanidine hydrochloride. The open circles indicate a Lorentzian fit (eq 3) of half-width $(2\Gamma/2\pi) = 6872$ Hz. It is clear that eq 3 accurately describes the spectrum of the photocurrent and from the measured half-width and eq 2 we determine $D_{20,w}$ to be 10.7×10^{-7} cm²/sec.

When Gdn·HCl is present in the scattering solutions, eq 1 no longer describes completely the spectrum of the scattered light, since the Gdn·HCl itself makes a small but observable contribution to the scattering. We may readily calculate this effect. The intensity of light scattered due to concentration fluctuations is proportional to the product of the molecular weight and concentration of the scatterer, as well as to the square of its refractive index increment, $\partial n/\partial c$ (Tanford, 1961). Since $\partial n/\partial c$ is about the same for lysozyme (Bruzzesi *et al.*, 1965), guanidine hydrochloride (Kielley and Harrington, 1960), and the chloride ion (Washburn, 1929) (roughly 1.9 cm³/g), we see that the ratios of the integrated intensity of the light scattered by lysozyme to that scattered by guanidine to that by chloride are given simply by their respective products of molecular weight and concentration. We thus obtain immediately that guanidine and chloride together scatter 3.5%/mol as much light as a 1% lysozyme solution. Hence, for a 6 M Gdn·HCl solution, the salt scatters 21% as much light as the lysozyme itself. If we treat guanidine and chloride as having the same diffusion coefficient D_G, and indicate the diffusion coefficient of lysozyme as D_L, then the spectrum of the scattered light is given from eq 1, where $\Gamma_L = D_L K^2$ and

$$S(\nu) \propto \frac{(\Gamma_L/2\pi)}{(\nu - \nu_0)^2 + (\Gamma_L/2\pi)^2} + (0.035)[\text{Gdn·HCl}]\frac{(\Gamma_G/2\pi)}{(\nu - \nu_0)^2 + (\Gamma_G/2\pi)^2} \quad (4)$$

$\Gamma_G = D_G K^2$, and where [Gdn·HCl] is the molar concentration of guanidine hydrochloride. In the self-beating spectrometer these two components in the scattered light will mix. The predominant terms in the self-beat spectrum of the photocurrent are given by (Dubin, 1970; Benedek, 1969; Cummins and Swinney, 1970)

$$S_i(\nu) \propto \frac{(2\Gamma_L/2\pi)}{\nu^2 + (2\Gamma_L/2\pi)^2} + 0.070[\text{Gdn·HCl}]\frac{(\Gamma_L + \Gamma_G)/2\pi}{\nu^2 + \{(\Gamma_L + \Gamma_G)/2\pi\}^2} \quad (5)$$

In effect, the lysozyme becomes a local oscillator (Dubin, 1970; Benedek, 1969; Cummins and Swinney, 1970) which enhances the observability of the Gdn·HCl scattering.

A modified Newton–Raphson (Margenau and Murphy, 1956) computer program was written to give a least-squares fit[3] of the data to eq 5. Fifty points, evenly spaced in frequency from approximately one-quarter the experimental half-width to about six times the experimental half-width, were employed in determining the best fit. In Figure 2 we display the self-beat spectrum (solid line) of the photocurrent for a Gdn·HCl concentration of 4.37 M. This spectrum was obtained at a temperature of 23.7° and a scattering angle of 62.9°. A best

[2] A detailed discussion of the relative merits of the multichannel wave analyzer approach and the autocorrelator methods has been presented by DeGiorgio and Lastovka (1971).

[3] This program was written by Dr. Joseph Lunacek, Department of Physics, University of California, Santa Barbara, Calif.

single Lorentzian fit (eq 3) with $(2\Gamma/2\pi) = 5077$ Hz is indicated by the open squares and clearly fails to describe adequately the observed spectrum. The best *two* Lorentzian fit which takes into account the scattering due to the guanidine hydrochloride (eq 5) is indicated by the open circles and fits the experimental data extremely well. From this fit the width of the self-beat term in eq 5 was determined as $(2\Gamma/2\pi) = 4505$ Hz and for the cross-beat term we obtained $(\Gamma_L + \Gamma_G)/2\pi = 22.39$ kHz. We were therefore able to determine D_G in addition to D_L.

We analyzed our data according to eq 5 for 31 concentrations of Gdn·HCl between 0 and 6.4 M. Typically three successive spectra were taken at each concentration of Gdn·HCl and the error bars on our determinations of $D_{20,w}$ for lysozyme are the spread in these results. The results obtained for the diffusion coefficient of lysozyme as a function of Gdn·HCl concentration are summarized in Figure 3. In addition, we obtained an average value of $D_{20,w}$ for guanidine hydrochloride of $(79 \pm 20) \times 10^{-7}$ cm²/sec.

In order to reduce the disulfide bridges of lysozyme, the protein was prepared in a 6.4 M Gdn·HCl solution which additionally contained 30 mM dithiothreitol. The action of the dithiothreitol further lowered the value of the diffusion coefficient ($D_{20,w}$) of the lysozyme to $(5.7 \pm 0.2) \times 10^{-7}$ cm²/sec. This value was obtained independent of the period of incubation of dithiothreitol between 90 min and 12 hr. In addition, heating the solution for 2 hr at 50° failed to change the value of $D_{20,w}$ from $(5.7 \pm 0.2) \times 10^{-7}$ cm²/sec.

It is important to note here that two factors made it possible to discern and account for the contribution of the guanidine hydrochloride to the total scattered light. Its very large diffusion coefficient (nearly an order of magnitude larger than that of lysozyme) made the spectrum of its contribution to the scattered light noticeably wider than that of the lysozyme itself. In addition the relative intensities of the light scattered by all species present could be readily determined from their known molecular weights and refractive index increments. Hence, only three adjustable parameters are required to obtain the best fit to eq 5, namely, Γ_L, Γ_G, and the zero frequency intercept for either of the two terms present in the equation. The zero frequency intercept of the other term is then fixed by the known relative intensities of the two terms.

It will be shown in the Discussion section that when two species do not have such dissimilar diffusion coefficients as those of lysozyme and Gdn·HCl, such a complete unraveling of the observed spectrum is not always feasible.

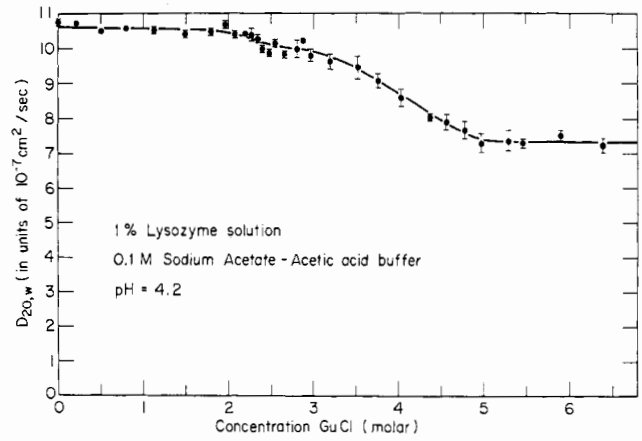

FIGURE 3: Effect of chemical denaturation on the diffusion coefficient of lysozyme. The diffusion coefficients were obtained from the spectrum of the light scatttered by buffered solutions of the enzyme (see Figures 1 and 2) and are corrected to standard conditions (20°, water).

Discussion

As shown in Figure 3, the diffusion coefficient of native lysozyme has a value of $(10.6 \pm 0.1) \times 10^{-7}$ cm²/sec. If this value of D is combined with a partial specific volume ($\bar{v}$) of (0.703 ± 0.004) cm³/g (Sophianopoulos *et al.*, 1962) and a sedimentation rate ($s_{20,w}$) of $(1.87 \pm 0.02) \times 10^{-13}$ sec (Sophianopoulos *et al.*, 1962; Colvin, 1952), one obtains the molecular weight of lysozyme as 14,500 ± 300. This is in excellent agreement with the value 14,600 as determined from the amino acid sequence of the molecule (Phillips, 1967). Our value of $D_{20,w}$ is also in good agreement with the value $(10.4 \pm 0.1) \times 10^{-7}$ cm²/sec (Colvin, 1952) in the concentration range 0.3–0.8% and $(10.6 \pm 0.2) \times 10^{-7}$ cm²/sec (Foord *et al.*, 1970) from 0.3 to 2.0%.

When Gdn·HCl is added in increasing concentrations we see from Figure 3 that the diffusion coefficient changes as expected from a denaturing process. Similar effects have been observed in the optical rotation (Tanford *et al.*, 1966) and the specific viscosity (Hamaguchi and Kurono, 1963). Above a Gdn·HCl concentration of 5 M, the diffusion coefficient of lysozyme is seen to plateau. The value of $D_{20,w}$ in this region is $(7.3 \pm 0.1) \times 10^{-7}$ cm²/sec. That is, the native form of the enzyme has a diffusion coefficient 45% greater than that of the denatured protein. The question of interest is: What is the nature of lysozyme for intermediate values of the concentration of guanidine hydrochloride? Does the molecule exist in two states for any value of the concentration of Gdn·HCl, the states having diffusion coefficients of $(10.6 \pm 0.1) \times 10^{-7}$ and $(7.3 \pm 0.1) \times 10^{-7}$ cm² per sec, respectively, or does lysozyme unfold gradually upon denaturation, with a single, smoothly varying diffusion coefficient?

In the first model we are dealing with a mixture of species, in the second with a single species. The classical macroscopic measurements such as optical rotatory dispersion and specific viscosity cannot distinguish between such models, whereas light scattering (as well as high resolution nuclear magnetic resonance) can in principle do so. A quantitative analysis (Dubin, 1970, 1972) of this problem unfortunately shows that in the specific case of lysozyme the self-beat spectrum of a mixture of the two different states of lysozyme would itself be essentially Lorentzian in profile, and hence one could not say that two species were present. Specifically, the solid line in Figure 4 displays the theoretically predicted self-beat spectrum of an equimolar mixture of two molecular species of the same molecular weight but differing in that one species has a diffusion coefficient 50% greater than that of the other. The open circles represent a *single* Lorentzian fit from which a value of D of 98% of the average of the two separate values of D is obtained. Clearly this is an excellent fit and hence, with our signal-to-noise ratios, it is impossible to distinguish this spectrum from a single Lorentzian. If we let $S_i(\nu)$ be the theoretically predicted self-beat spectrum and $F(\nu)$ be the best *single* Lorentzian fit to this spectrum we may then define the normalized percentage rms error of the fit to the spectrum for N discrete frequencies ν_j as

$$\% \text{ rms error} = 100\frac{\left[\frac{1}{N}\sum_{j=1}^{N}\{S_i(\nu_j) - F(\nu_j)\}^2\right]^{1/2}}{F(0)} \quad (6)$$

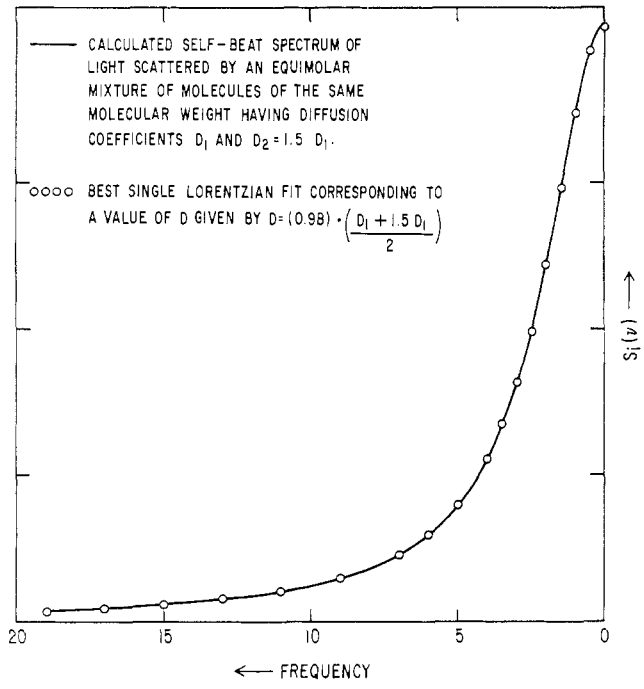

FIGURE 4: Self-beat spectrum of the light scattered by a two-component mixture of molecules differing in their diffusion coefficients by 50%. The solid line is the theoretically predicted spectrum and the open circles represent the best *single* Lorentzian fit. Note the negligibly small deviation of the single Lorentzian fit from the theoretically predicted spectrum. The vertical scale is in arbitrary units. The horizontal scale is adjusted such that a single species with diffusion coefficient D_1 would give a spectrum with a half-width of two units.

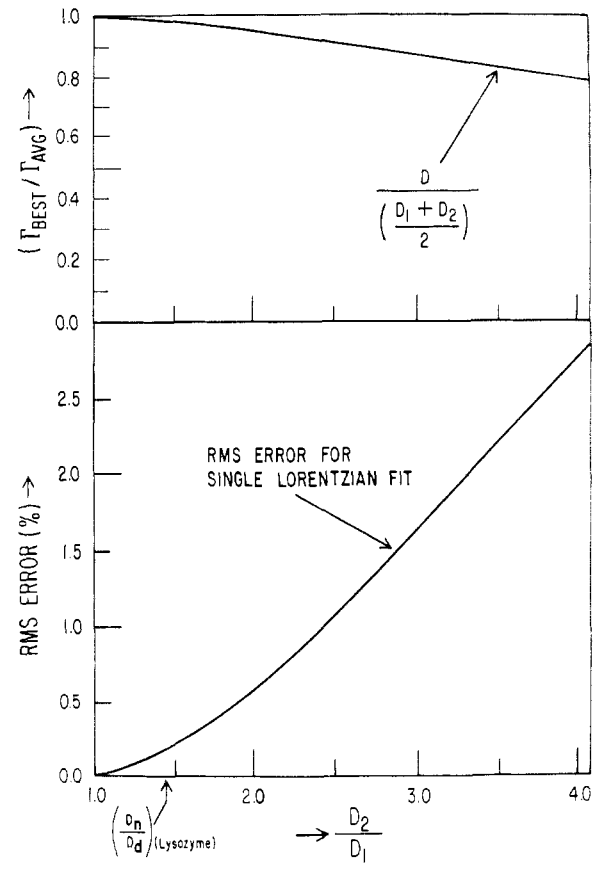

FIGURE 5: Width and normalized per cent root-mean-square deviation (see eq 6) of the best *single* Lorentzian fit to the self-beat spectrum of the light scattered by various equimolar mixtures of macromolecules differing only in diffusion coefficient. For lysozyme the ratio D_n/D_d was determined to be 1.45 (see Figure 3) as indicated by the arrow on the abscissa. For this ratio the single Lorentzian fit described adequately all our results.

Employing eq 6 the percentage rms deviation of the best single Lorentzian fit as shown in Figure 4 is only 0.2%. Similar theoretical calculations have been done for various equimolar mixtures of molecules whose diffusion coefficients differ by up to a factor 4. These calculations are summarized in Figure 5. The upper graph shows how the width of the best *single* Lorentzian fit becomes progressively pulled below the average of the two separate values, while the lower curve demonstrates that this best fit remains remarkably Lorentzian even for D_2/D_1 greater than 2. The difference in diffusion coefficient must become quite substantial for the observed self-beat spectrum to appear non-Lorentzian. Ironically, the self-beat spectrum is even more Lorentzian than the spectrum of *light* scattered by the mixture (Dubin, 1970). This arises because the cross-beat term present in $S_i(\nu)$ has a width equal to the average of the two self-beat terms present. Hence, one might suspect that the heterodyne mixing spectrometer (Benedek, 1969; Cummins and Swinney, 1970), which does not produce the cross-beat term, might be more suitable for resolving a non-Lorentzian spectrum. While this suspicion is indeed correct (Dubin, 1970), the improvement in detectability of non-Lorentzian character in the observed spectrum is too small (Dubin, 1970) to warrant the further complexities involved in the heterodyne technique. In summary, if the two species present differ in diffusion coefficient by less than about a factor of 3, it is not readily possible to determine from the self-beat spectrum of the scattered light that there are indeed two species present. On the other hand, given that one knows from other considerations that there are two species present, and then obtains the diffusion coefficients of these species separately, it is a straightforward procedure to determine the relative fraction of each species when both exist together in a mixture.

In the present case, the diffusion coefficients of the two species differ by only 45%. We therefore cannot determine from these results whether the lysozyme molecule unfolds progressively as a function of Gdn·HCl concentration or whether only the native and denatured forms exist at any value of Gdn·HCl concentration with just the relative fraction of each changing. However, the nuclear magnetic resonance experiments of Glickson *et al.* (1968) indicate that the latter process appears to describe the denaturation of lysozyme. If we accept this, then it is clear from Figure 5 that the measured value of D is accurately the number-weighted average of the diffusion coefficients of the native and denatured molecules present for any value of Gdn·HCl concentration. That is, if F_n is the fraction of native lysozyme which remains for any value of the concentration of guadinine hydrochloride, and D_n and D_d are the diffusion coefficients of native and denatured lysozyme respectively, then the value of the diffusion coefficient D obtained by treating the observed self-beat spectrum as a single Lorentzian curve is given by

$$D \approx F_n D_n + (1 - F_n) D_d \tag{7}$$

and we thus obtain

$$F_n \approx (D - D_d)/(D_n - D_d) \tag{8}$$

From Figure 3, we find that D_n and D_d are given as $(10.6 \pm 0.1) \times 10^{-7}$ and $(7.3 \pm 0.1) \times 10^{-7}$ cm² per sec, respectively. Combining these values of D_n and D_d with those of D in Figure 3 we may employ eq 8 to determine the fraction of native lysozyme and denatured lysozyme as a function of Gdn·HCl concentration. For example, at a Gdn·HCl concentration of 3.8 M, the value of $F_n = {}^1/_2$, *i.e.*, equal numbers of native and denatured lysozyme molecules are present at this concentration.

We now turn to the problem of the size of the native and denatured lysozyme molecules. Employing the value of the translational diffusion coefficient quoted here, and combining this with the value of the rotational diffusion coefficient of lysozyme as measured by depolarized light-scattering spectroscopy, Dubin *et al.* (1971) determined that native lysozyme in solution is hydrodynamically equivalent to a prolate ellipsoid of revolution of major axis ($2a$) and minor axis ($2b$) 55 and 33 Å, respectively. These values include a uniform shell of hydration about 3.5 Å thick. Unfortunately, the rotational diffusion coefficient of the denatured lysozyme has not yet been measured, preventing a similar size and shape determination for the fully denatured protein molecule. However, if we consider the enzyme represented simply as a sphere, our value of $D_{20,w}$ for the native molecule implies, *via* the Stokes–Einstein equation, that native lysozyme is hydrodynamically equivalent to a sphere of diameter 40.3 Å which compares favorably with the dimensions obtained by Dubin *et al.* (1971). The value of the specific viscosity of lysozyme of 3.3 cm³/g obtained by Hamaguchi and Kurono (1963) indicate that the sphere hydrodynamically equivalent to lysozyme has a diameter of 39.4 Å, again in good agreement with the other determinations. If we now consider our value of $D_{20,w}$ for the denatured enzyme, we obtain the hydrodynamically equivalent sphere as having a diameter of 58.5 Å. This is in excellent agreement with the specific viscosity data of Hamaguchi and Kurono (1963) who obtain 8.7 cm³/g as the specific viscosity of denatured lysozyme, from which the diameter of the equivalent sphere may be deduced as 54.5 Å. Hence, it is clear that upon denaturation, lysozyme retains a compact configuration, and the change in diffusion coefficient and specific viscosity of the enzyme reflects an effectively isotropic swelling of the molecule. If the protein's disulfide bridges are reduced by dithiothreitol the diameter of the equivalent hydrodynamic sphere is 75 Å as deduced from its value of $D_{20,w}$ of $(5.7 \pm 0.2) \times 10^{-7}$ cm²/sec. Hence, removal of the disulfide bridges causes additional loss of ordered structure in lysozyme. The results on lysozyme are in contrast with those of Rimai *et al.*, (1970) who studied the denaturation of ribonuclease, an enzyme of nearly the same molecular weight as lysozyme. Using essentially the same experimental techniques described in this paper, these workers observed a surprising large (greater than an order of magnitude) change in the value of D for ribonuclease upon denaturation. However, the state of partial aggregation of the samples of ribonuclease employed in that study must be considered when interpreting their results.

In conclusion, we have shown that optical mixing spectroscopy provides a convenient tool for the study of the denaturation of proteins by measuring changes in their translational diffusion coefficients. Our data indicate that on chemical denaturation lysozyme increases by about 45% in linear dimension while retaining a compact globular structure. The spectral line shapes alone do not permit a distinction between different models of lysozyme denaturation because of the relatively small change in diffusion coefficient. However, using nuclear resonance measurements (Glickson *et al.*, 1968) which favor the two-state model, our data permit the deduction of the relative proportion of lysozyme in each state as a function of Gdn·HCl concentration. Finally, we may observe that the rotational diffusion coeffcient (D_R) of lysozyme can be measured from the spectrum of the depolarized scattered light (Dubin *et al.*, 1971). By measuring the rotational as well as the translational diffusion coefficient in the fully denatured state, it will be possible to deduce the hydrodynamic shape of the denatured lysozyme. Furthermore, since D_R varies as the cube of the linear molecular dimension, the depolarized spectrum in partly denatured states might permit a distinction between the different models for protein denaturation.

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Hydrogen-Bonded Complexes of Adenine and Thymine Nucleoside Alkyl Phosphotriesters in Deuteriochloroform†

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ABSTRACT: The association of neutral analogs of adenine and thymine dinucleoside monophosphates in deuteriochloroform was investigated by infrared spectroscopy. This is the first time that such studies have been done at the dimer level; because of solubility requirements, such experiments could be done only when nucleoside alkyl phosphotriesters became available. Association constants for both self-association and cross-association were determined by analysis of divergence from Beer's law of NH and NH_2 stretching bands. The complementary dimers associated with an intrinsic constant of 192 M^{-1} as compared to a value of 92 M^{-1} for the corresponding monomers. The relatively low value for the dimers indicates that the association process is not cooperative. The two residues of the dimers behave nearly independently in the association process; therefore, the restriction on the rotation about the bonds of the backbone must be small. This conclusion is discussed in relation to recent reports on unperturbed dimensions of polynucleotides in aqueous solution. The results reinforce the conclusion from the studies on polynucleotides that the restriction of the rotation of the backbone of a polynucleotide in solution cannot be due to steric hindrance alone. Our data support the suggestion that the electrostatic interaction between the charged groups of the polynucleotide backbone and that between the charged groups and water could be the cause of the rigidity of the polynucleotide in a random coil conformation observed in aqueous solution.

Hydrogen bonding of purine and pyrimidine bases and nucleosides in organic solvents has been studied by a variety of methods in several laboratories. Only pertinent references concerning adenine and thymine (or uracil) association are cited here (Hamlin *et al.*, 1965; Kyogoku *et al.*, 1966, 1967a,b, 1969; Katz and Penman, 1966; Katz, 1969; Nagel and Hanlon, 1972). Most of these studies were done in nonpolar organic solvents in order to minimize solvent competition for hydrogen-bonding sites and to eliminate hydrophobic stacking interactions. The reason for such studies is to try to understand the origin of the specificity in the basic pairing scheme in polynucleotide complexes in terms of "electronic complementarity" (Kyogoku *et al.*, 1969).

In the present study, we were able to examine dimer–dimer association in chloroform for the first time because of the availability of alkyl phosphotriesters. With suitable blocking groups on the sugar hydroxyls, these compounds are readily soluble in chloroform. The two residues of the dimers were observed to behave almost independently in the association process; hence, there is little restriction of rotation about the bonds in the backbone. The relationship of these findings to recent studies on unperturbed dimensions of polynucleotides in aqueous solution is discussed.

Materials

1-Cyclohexylthymine (T) and 9-ethyladenine (A) were purchased from Cyclo Chemical Co. Deuteriochloroform (99.8%) from Merck and Co. was dried over type 4A molecular sieves before use. All compounds used for hydrogen bonding were dried for 3 hr in an Aberhalden with refluxing acetone in the presence of P_2O_5 on aspirator vacuum.

Chemical Synthesis of the Phosphotriesters

General Strategy. The protected thymidine triester, Tp(Et)T, was prepared by condensation of a protected nucleoside (MTrT) with the ethyl ester of the protected nucleoside 5′-phosphate (d-EtpTOAc) followed by purification by silica gel chromatography. The preparation of the protected adenine triester, dAp(Et)dA, involved first synthesis of the fully protected triester d-DMTrA^{Bz}p(C_2H_5)A$^{(Bz)_2}$OBz. The *N*-benzoyl groups were then selectively removed by treatment with hydrazine hydrate (Letsinger *et al.*, 1968).

Analytical Procedures. Thin layer chromatography was carried out on Eastman 6060 chromatogram sheets. Paper chromatography was performed *via* the descending technique on Whatman 3MM paper using the following solvent sys-

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