Characterization of Heterogeneous Solutions Using Laser Light Scattering: Study of the Tubulin System†

Jon S. Gethner,‡ George W. Flynn, Bruce J. Berne, and Felicia Gaskin*

ABSTRACT: Laser light scattering has been applied to a systematic study of a heterogeneous solution of tubulin at low temperature—conditions under which tubulin assembly into microtubules does not take place. Methods of analyzing laser light scattering results obtained from solutions containing multiple components are discussed. Data analysis techniques are described and their application to the determination of diffusion constants from experimental data is extensively illustrated. Multiple components were found under the conditions that the tubulin was studied. We have identified one component having $D_{20,w} = 4.41 \times 10^{-7}$ cm^2/s ($\sigma = 0.54 \times 10^{-7}$ cm^2/s) which has the expected value for tubulin dimer. In addition, we have found two components which are significantly larger than tubulin. One large component has $D_{20,w} \sim 0.55 \times 10^{-7}$ cm^2/s and is present in all samples at 4 °C even after centrifugation to remove components greater than 10 S. Another large component having 3.2×10^{-7} cm^2/s $\geq D_{20,w} \gtrsim 1.5 \times 10^{-7}$ cm^2/s has been found to sediment with 10 S $\leq s <$ 20 S.

Recently, the new technique of laser light scattering has been used to accurately measure the hydrodynamic diffusion constants of molecules which are small compared with the wavelength of visible light (Chu, 1970; Pecora, 1972; Berne and Pecora, 1974). While extensive work has recently been performed in which diffusion constants of biological macromolecules have been measured, most systems studied have been prepared to contain one relatively pure component. Many interesting biochemical systems, however, cannot be prepared to contain a single pure component, either due to strictly preparative considerations or due to the presence of complex equilibria in solution. In this paper laser light scattering is applied to a systematic investigation of tubulin preparations prepared by the recyclization procedure of Shelanski et al. (1973). This tubulin preparation is a complex, multicomponent protein system and contains 80% tubulin predominantly in the form of 6S dimer and 36S rings (Gaskin et al., 1975). Methods of analyzing data obtained from heterogeneous[1] solutions are utilized so that the diffusion constants of each component present may be estimated. In the accompanying paper (Gethner et al., 1977), we consider the existence of a tubulin-ring or tubulin-aggregate equilibrium.

Experimental Section

Preparation of Tubulin. Porcine tubulin was purified from fresh brains following the recyclization procedure developed by Shelanski et al. (1973). On the day of the experiment, tubulin which had been stored at −20 °C in 4 M glycerol in running buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.55, and containing 10^{-3} M EGTA[2] and 5×10^{-4} M $MgCl_2$] was dialyzed for 3 h at 4 °C against 250 vol of running buffer. Protein concentration after dialysis was estimated using $A_{280} = 0.89$ for a sample containing 1 mg/mL. Samples were adjusted to a fixed concentration of 2.2 mg/mL by the addition of fresh running buffer after dialysis. For concentration dependent studies samples were diluted further with running buffer. GTP was added to a concentration of 10^{-3} M.

The above protein sample was used to prepare the following samples.

(i) HSX (<20 S) samples were prepared by centrifuging 1.0 mL of tubulin at 4 °C for 1 h using a Beckman type 40 rotor operated at 35 000 rpm. The clearing factor for these conditions is 20 (C. R. McEwen, private communication). Approximately 0.5 mL of the sample was withdrawn from the centrifuge tube using a precooled 1-cm^3 disposable syringe. Care was taken to avoid touching the syringe on the walls of the tube. Solution was not withdrawn from the region of the pellet or the meniscus. Samples were drawn into the syringe slowly to minimize denaturation, transferred into a precooled fluorescence cell, and sealed with a Teflon stopper. The sample was stored at 4 °C or in an ice bath until correlation functions were run.

(ii) HSX (<10 S) samples were prepared by centrifuging 0.8 mL of tubulin at 4 °C for 12 h using a Beckman SW 50.1 rotor at 35 000 rpm. Approximately 0.4 mL of solution was withdrawn and stored as described above.

(iii) DEAE samples were prepared by purifying recycled tubulin on a 1.5 cm × 25 cm DEAE-Sephadex A-50 column which had been equilibrated with buffer containing 0.3 M KCl. Tubulin in 2 M glycerol and 0.15 M KCl was applied to the

† From the Department of Chemistry, Columbia University, New York, New York 10027 (J.S.G., G.W.F., and B.J.B.), and the Departments of Pathology (Neuropathology) and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461 (F.G.). *Received February 2, 1977; revised manuscript received September 8, 1977.* Portions of this work have been presented at meetings of the American Physical Society (New York, February 1976) and the Biophysical Society (Seattle, February 1976). This work was taken, in part, from the Ph.D. Dissertation of Jon S. Gethner, Department of Chemistry, Columbia University, 1976. Support for the work was from grants from the National Science Foundation (NSF-CH-76-02414), the U.S. Public Health Service (NS-12418), and a Research Career Development Award to F.G.

‡ Present address: Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014.

[1] The term heterogeneous solution is being used specifically to refer to a solution containing a distribution of molecules each component of which is a distinctly different species (e.g., monomer, dimer). This is to be distinguished from a polydisperse solution in which a single well-defined component (e.g., monomer) may not be characterized by a unique molecular weight. Each component within a heterogeneous solution may be polydisperse.

[2] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; BLF, baseline fit method; BLM, baseline monitor method.

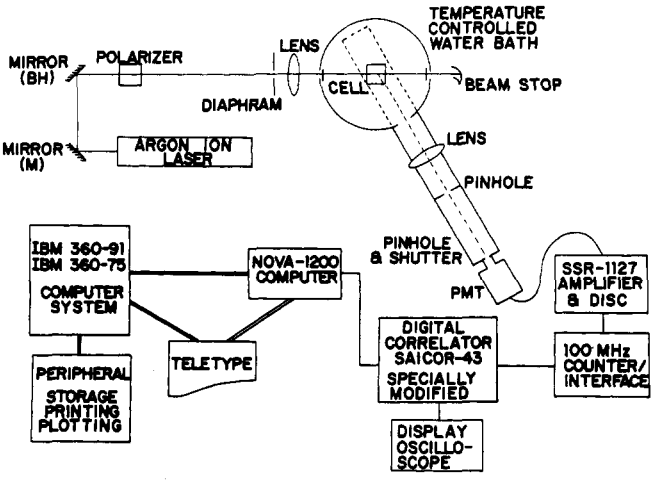

FIGURE 1: Block diagram of the apparatus. Mirrors M and BH are front surfaced aluminum reflectors which are flat to $\lambda/10$. BH is a beam steering device (Newport Research Corp.) which is used to bring the laser beam to the proper height. Using a $\frac{1}{4}$ wave plate (polarizer in Figure 1), the polarization of the incident light is rotated so that it is perpendicular to the scattering plane. The laser light is focused using an achromatic lens having $f = 14$ cm and enters a cylindrical water bath normal to a quartz entrance window. Scattered light is imaged onto a 500-μm pinhole using a lens having $F = 10$ cm. A second pinhole of 500 μm in front of the photomultiplier tube restricts the number of coherence areas collected to approximately 1.4. Photon counting techniques are used to provide a pulse input to a digital counter/digital correlator analysis system. A Channeltron photomultiplier tube having a bialkali photocathode of 3-mm diameter was operated at -1630 V. Under these conditions the dark count rate is less than 5 counts/s at room temperature and correlated after pulsing is negligible (Gethner and Flynn, 1975).

column. The first fraction eluted with 0.3 M KCl was discarded. The column was then eluted with 0.8 M KCl and collected in 2-mL fractions. The two peak fractions (based upon A_{280}) were centrifuged separately under conditions described for preparing a HSX (<20S) sample and used for light scattering determinations.

Protein concentrations were determined after completion of the light scattering by the method of Lowry et al. (1951).

Electron Microscopy. One drop of sample was applied to a Formvar grid for 10 or 30 s (either at 4 °C or at 27 °C). After rinsing with 6 drops of 1% uranyl acetate, the grid was blotted dry with filter paper.

Materials. 2-(*N*-Morpholino)ethanesulfonic acid was Calbiochem Grade A. GTP (type II-S) was from Sigma. All other chemicals were standard reagent grade. All solutions used for laser light scattering which were not centrifuged were filtered through 0.22-μm Millipore filters. Distilled water used for cleaning cells was filtered through 0.22-μm Millipore filters.

Gel Analysis. Gel analysis was performed on 5% polyacrylamide gels with 0.1% sodium dodecyl sulfate and 8 M urea (Feit et al., 1971). Staining was with fast green (Gorovsky et al., 1970). After centrifugation, all samples of tubulin (<20 S and <10 S) also contained high molecular weight components of about 350 000 as reported by Gaskin et al. (1974) for the standard tubulin preparation.

Sucrose Gradients. Linear (5–20%) sucrose gradients in running buffer containing 1 mM GTP were prepared in cellulose nitrate centrifuge tubes which had been previously selected for optical uniformity. Tubulin HSX, (<20 S), 0.2 mL, was layered onto the top of the gradient. The gradients were centrifuged at 4 °C using a Beckman SW 50.1 rotor operated at 44 000 rpm for 5 h. Laser light scattering measurements were performed using a procedure similar to that described by Koppel (1973) who separated ribosomal subunits on a sucrose gradient. Gradients were dripped and analyzed for protein concentration after completion of the light scattering.

Laser Light Scattering Measurements. Laser light scattering measurements were performed using the spectrometer shown in the block diagram of Figure 1. The spectrometer has been described elsewhere (Gethner, 1976). Spectra were obtained by scattering light of 488 nm from a Spectra-Physics Model 165 argon ion laser which was amplitude stabilized and operated on a single longitudinal mode. Wavelength independence was checked for several samples. The maximum laser power used was, in most cases, 10 mW. The sample, contained in a sealed fluorescence cuvette, was at the center of a cylindrical water bath which was controlled to ± 0.2 °C by a thermostatically regulated liquid circulator (Brinkman Lauda K2R). A Channeltron photomultiplier (bialkali) was used to provide a pulse input to a 3-bit counter and a modified Saicor 43-A digital autocorrelator (Gethner, 1976). Autocorrelation functions were analyzed to determine diffusion constants on an IBM 360/91 computer system using least-squares regression techniques (Gethner, 1976).

The light scattering from a homogeneous solution of Brownian particles which are small relative to the wavelength of light has been extensively treated both theoretically and experimentally (Berne and Pecora, 1976; Cummins and Swinney, 1970; Benedek, 1968). The experimentally measurable quantity in our experiments is the homodyne intensity autocorrelation function of the scattered light:

$$C(t) = \langle I(0)I(t)\rangle = \langle I(t)\rangle^2\left[1 + \frac{1}{N_c^2}|F_s(q,t)|^2\right]$$

$I(t)$ is the intensity of light observed at the detector at a time, t. $\langle\rangle$ indicates a time averaged quantity. N_c is the number of coherence areas which the detector observes and is an instrumental constant. $F_s(q,t)$ is the single particle scattering function and for the case of a dilute solution of Brownian particles is given by (Berne and Pecora, 1976)

$$F_s(q,t) = e^{-q^2Dt}$$

where D is the particle self-diffusion constant and $q = 4\pi n/\lambda \sin(\theta/2)$. n is the solution index of refraction, λ is the wavelength of the incident light, and θ is the scattering angle.

Heterogeneity may be introduced by averaging $F_s(q,t)$ over a distribution function describing the heterogeneous ensemble. Since only particles with different diffusion constants may be detected, it is convenient to express this average over a distribution of diffusion constants.

$$F_s(q,t) = \frac{\int_0^\infty \alpha^2(D)S(q,D)e^{-q^2Dt}P(D)\mathrm{d}D}{\int_0^\infty \alpha^2(D)S(q,D)P(D)\mathrm{d}D}$$

where $\alpha(D)$ is the polarizability of the particle having a diffusion constant D. $S(q,D)$ is the single particle structure factor and $P(D)$ is distribution function describing the particles. The distribution may not be unique in that there may be more than one distinct component having the same D. Assuming $P(D)$ to be composed of a finite number of δ functions at various values of D corresponding to a distribution of many different particles each of which is perfectly monodisperse, we obtain

$$F_s(q,t) = \frac{\sum_i \alpha^2(D_i)S(q,D_i)e^{-q^2D_it}N(D_i)}{\sum_i \alpha^2(D_i)S(q,D_i)N(D_i)}$$

where $N(D_i)$ is the number of particles having the diffusion constant D_i. Since the product $\alpha^2(D_i)S(q,D_i)N(D_i)$ will always appear together, $S_i(q)$ is defined as the product and

$$F_s(q,t) = \sum_i S_i(q)e^{-q^2D_it} \Big/ \sum_i S_i(q)$$

In many instances, the scattering from a heterogeneous solution will be due primarily to just two components either because of the relative concentrations of the components, $N(D_i)$, or the relative polarizability factors, $\alpha(D_i)$, or both. Thus $F_s(q,t)$ may be specialized for two components to obtain

$$F_s(q,t) = \frac{e^{-q^2D_1t} + A(q)e^{-q^2D_2t}}{1 + A(q)}; \quad A(q) = \frac{S_2(q)}{S_1(q)}$$

Expanding each of the exponentials in a Taylor series, we obtain an exponential correct to terms through t^2 which is valid at *short times* (Hocker et al., 1973)

$$F_s(q,t) = \exp -[q^2\overline{D}t - \tfrac{1}{2}(\overline{D^2} - \overline{D}^2)q^4t^2 + \ldots]$$

where

$$\overline{D} = \frac{\sum_i S_i(q)D_i}{\sum_i S_i(q)} = \frac{D_1 + A(q)D_2}{1 + A(q)}$$

and

$$\overline{D^2} = \frac{\sum_i S_i(q)D_i^2}{\sum_i S_i(q)} = \frac{D_1^2 + A(q)D_2^2}{1 + A(q)}$$

A computer fit of ln $[F_s(q,t)]$ to the series $A - B_1t + B_2t^2$ for small t will give

$$B_1 = q^2\overline{D}$$

and

$$B_2 = \tfrac{1}{2}q^4(\overline{D^2} - \overline{D}^2)$$

$\overline{D}$ measured in this manner is seen to be a weighted diffusion constant. For $D_2 < D_1$ and $A(q) > 0$, $\overline{D} < D_1$. B_2 is like a weighted "variance" of the distribution of the diffusion constants of the particles. This analysis is essentially the same as the cumulant analysis introduced by Koppel (1972) with a redefinition of B_1 and B_2. We will, therefore, refer to such an analysis of the data as a cumulant analysis.

Baselines were estimated by two methods. The correlator computes the average intensity of the light giving the true infinite time value of the baseline. Correlation functions normalized using this value are referred to as being normalized using the baseline monitor method (BLM). If very large particles of denatured protein or dust are present or some other effect occurs which results in a very long slow decay, then the average value of the correlation function at some long delay time may be significantly greater than the BLM value of the baseline. If the decay of the "baseline" is sufficiently slow, a good estimate of the normalization may be taken to be the $t = 0$ intercept obtained by performing a linear least-squares fit to the long time points of the correlation function. This has been done with points 300 to 398 of the 398 point correlation function. The correlator time base is adjusted so that point 350 is at least 7 correlation times of the fastest exponential decay contained in the correlation function. Correlation functions normalized in this way are referred to as being normalized using a baseline fit method (BLF). A discussion of the types of errors which arise from the two normalization schemes appears elsewhere (Gethner, 1976).

Data Evaluation Considerations. The correlation function of a typical sample is shown in Figure 2. The correlation function shown is obviously not described by a single exponential decay. Figure 3 shows a correlation function obtained under conditions more favorable to the observation of the multiple exponential behavior. Data similar to that shown in Figure 3 may be analyzed in terms of two exponential decays. The slow rate drawn in Figure 3 is estimated by drawing a straight line through the long time points. The slight upward curvature from the line drawn is typical for experimental points late in the decay curve which are difficult to distinguish from the baseline. After the slow rate is determined, the slow exponential may be subtracted from the initial points in order to obtain the fast rate. The fast rate shown in Figure 3 has been obtained from a second-order cumulant analysis. The diffusion constant obtained is an average and may be deconvoluted by methods described above. After deconvolution the diffusion constant of the smallest component (largest diffusion constant) is the same as that obtained when the slow diffusion constant is first subtracted from the correlation function and the resulting curve is analyzed. The slow diffusion constant can be subtracted only when the two exponentials are clearly resolved, however. We have therefore used the cumulant analysis technique to calculate $\overline{D}$. The first rate is then approximated by correcting $\overline{D}$ using B_2, the normalized value of the second cumulant. The correction is (Hocker et al., 1973)

$$D^f = \overline{D}\left(1 + B_2 + \frac{(B_2)^2}{4}\right)$$

When the two exponentials are not resolved, such as in Figure 2, D^f will be accurate to within approximately 10% (providing the estimate of B_2 is accurate) (Doherty and Benedek, 1974). When resolved the slow rate is used to verify the value of D^f.

Several HSX (<20S) solutions were observed both by homodyne detection and heterodyne detection. No difference in the observed diffusion constant was found. The agreement of those two methods rules out the possibility of a systematic error in the baseline due to number fluctuations (Schaefer and Berne, 1972). These results also indicate that sources of accidental heterodyne power in the homodyne experiment must be negligible. No power dependence was observed when levels greater than 10 mW were used.

Diffusion Constants of Tubulin Preparations

Since our tubulin preparations were known to contain 6S and 36S components in the analytical ultracentrifuge at 4 °C, experiments were performed to look at preparations in which 20S and 10S components were removed by centrifugation. Our electron microscopy showed no specific aggregates such as double rings in the <20S and <10S samples. As expected the tubulin preparations before centrifugation contained double rings and spirals which had been previously reported (Kirschner et al., 1974; Erickson, 1974).

HSX (<20S) Preparation. $\overline{D}_{20,w} = 1.61 \times 10^{-7}$ cm^2/s ($\sigma = 0.05 \times 10^{-7}$ cm^2/s) was obtained for the HSX (<20S) sample (see Table I). Figure 2 is a curve typical of those obtained in this series of runs. The marked deviation of the correlation function from single exponential behavior even at short times is indicative of a highly heterogeneous sample.

Under several experimental conditions used for analyzing HSX (<20S) samples, two exponentials were observed (see

TABLE I: Average $\overline{D}_{20,w}$ for Tubulin Preparations.

Solution	Concn (mg/mL)	History	$\overline{D}_{20,w} \times 10^7$ [a]	No. of runs [b]	No. of preps
HSX (<20S)	~1	HSX <20 S; 4 °C	1.61 ± 0.05	20	2
HSX (<10S)	0.45	HSX <10 S; 4 °C	3.33 ± 0.80	4	2
DEAE	~1	Column separation, HSX <20 S; 4 °C	1.38 ± 0.30	2	1

[a] The error indicated is one standard deviation of the mean. Due to the large number of correlation functions measured, this error is usually small. Any single correlation function may deviate from the average by up to ±25%. The error is strongly correlated to the quality of the baseline used. This has been discussed in Gethner (1976). [b] Each run consisted of the measurement and analysis of at least three correlation functions.

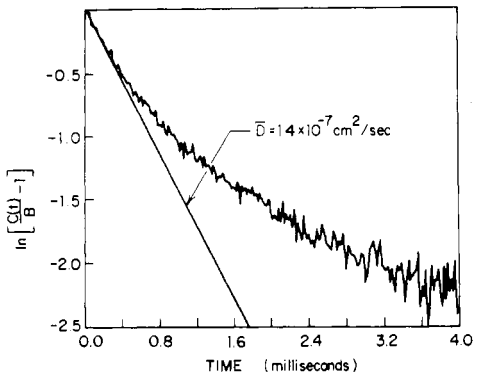

FIGURE 2: HSX (<20S) sample; logarithm of the normalized autocorrelation function. $\theta = 35°$, $T = 4.2$ °C, $\lambda = 488$ nm. Two hundred points (connected by lines) are shown at 20 μs/point. The correlation function was accumulated for 3×10^6 sums and normalized to the BLM value. The straight line drawn is the computed fit for $\overline{D}$ (see text). Correction of $\overline{D}$ to standard conditions gives $D_{20,w} = 2.25 \times 10^{-7}$ cm²/s. The marked curvature from the line drawn is indicative of a heterogeneous solution in which the exponentials from the individual components are not resolvable. BLF normalization or allowing the baseline to be a variable does not result in a significant improvement in the fit. The difference between $D_{20,w}$ and the average value for similar solutions given in Table I is indicative of the variation of the data between individual runs and sample preparations as well as the large number of correlation spectra used to compute the average given in Table I.

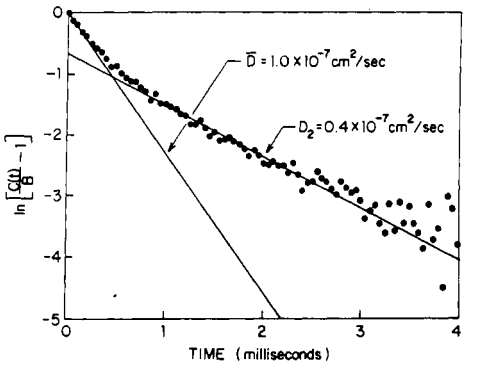

FIGURE 3: HSX (<20S) sample; logarithm of the normalized autocorrelation function showing the resolution of two components. $\theta = 35°$, $T = 4.2$ °C, $\lambda = 488$ nm. Sixty points are shown at 50 μs/point. The correlation spectrum was accumulated for 7.5×10^5 sums resulting in a baseline (BLM) of 67 354 counts. Twenty milliwatts of power was used. D_2 is the diffusion constant of the large component (see Experimental Section) and was obtained by a linear least-squares fit to a subset of points between 1.2 and 2.2 ms. $\overline{D}$ was obtained by computer fitting techniques described in the text. Correction of $\overline{D}$ and D_2 to standard conditions gives $D_{20,w} = 1.7 \times 10^{-7}$ cm²/s and D_2 ($T = 20$ °C) $= 0.66 \times 10^{-7}$ cm²/s. Deviation of these values from the averages shown in Table I is indicative of the experimental error in an individual determination. The signal-to-noise ratio for the correlation function is better than that observed in Figure 2 primarily due to the long time per channel and high laser power. This is discussed further in the Experimental Section.

Figure 3). Analyzing several sets of data results in a value of $D_{20,w} = 0.62 \times 10^{-7}$ cm²/s ($\sigma = 0.09 \times 10^{-7}$ cm²/s) for the diffusion constant of the large component and $D_{20,w}^{f} = 3.21 \times 10^{-7}$ cm²/s ($\sigma = 0.57 \times 10^{-7}$ cm²/s) for the small component. There was no apparent time dependence of $\overline{D}$ between 30 min and 6 h after centrifugation of the tubulin samples. Within experimental error there was no concentration dependence of the diffusion constant measured on a HSX (<20S) sample for protein concentrations in the range 0.2 to 0.8 mg/mL.

No angular dependence was found for $\overline{D}$ or D^f measured on HSX (<20S) samples. There should be no angular dependence of the diffusion constant obtained from a solution of homogeneous particles undergoing Brownian motion. The angular independence of $\overline{D}$ means that the single particle scattering factors of the individual components must be similar (see Laser Light Scattering Measurements). This result implies that all the components are either small relative to the wavelength of light or are roughly the same size and shape as one another.

The angular dependence of the correlation time (τ_c) was determined over the range 35° to 130°. The plot of τ_c vs. q^2 was a straight line with zero intercept. The value of $\overline{D}$ determined from the slope was in agreement with $\overline{D}$ obtained from the individual measurements.

HSX (<10S) Preparation. $D_{20,w} = 3.33 \times 10^{-7}$ cm²/s ($\sigma = 0.80 \times 10^{-7}$ cm²/s) was found for HSX (<10S) tubulin preparations (Table I).

Under optimum signal-to-noise conditions, two exponential decays were clearly visible. One such correlation function is shown in Figure 4. Analysis of the slow decay gives $D_{20,w} = 0.48 \times 10^{-7}$ cm²/s for the large component. Analysis of the HSX (<10S) data to estimate the diffusion constant corresponding to the fast decay rate gives $D_{20,w}^{f} = 4.41 \times 10^{-7}$ cm²/s ($\sigma = 0.54 \times 10^{-7}$ cm²/s). To within experimental error $D_{20,w}^{f}$ is that expected from a solution of tubulin (dimer).

Other Methods of Tubulin Preparation. In order to ensure that the results obtained were not artifacts of our sample preparation procedure, tubulin samples were prepared for the light scattering measurements by two other techniques. Tubulin prepared by DEAE column purification had a value of $\overline{D}$ (see Table I) of $1.38 \pm 0.30 \times 10^{-7}$ cm²/s.[3] This value apparently reflects the presence of large amounts of denatured or aggregated protein.

Light scattering experiments performed on protein which had been separated on a linear sucrose gradient suggested that pure tubulin (dimer) was not present in the slow peak. These

[3] The error limits encompass the maximum and minimum values for D obtained in the set of measurements performed.

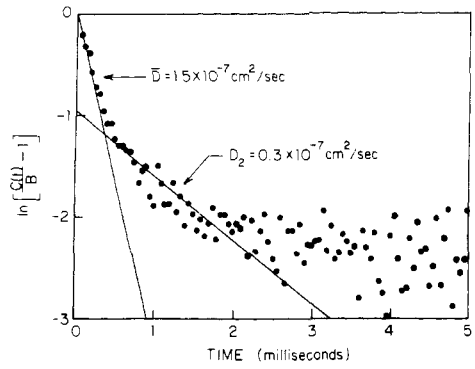

FIGURE 4: HSX (<10S) sample; logarithm of the normalized autocorrelation function. $\theta = 35°$, $T = 4.0$ °C, $\lambda = 488$ nm. Forty points are shown at 50 μs/point. The correlation spectrum was accumulated for 3×10^6 sums resulting in a baseline (BLM) of 10 206 counts. Twenty milliwatts of laser power was used. D_2 is the diffusion constant of the large component (see Experimental Section) and was obtained by drawing a straight line by hand. $\bar{D}$ was obtained by computer fitting to a power series in time (as described in the text) and by a nonlinear least-squares fit to a function containing two exponential decays of undetermined amplitude and an undetermined baseline. Correction of $\bar{D}$ and D_2 to standard conditions gives $D_{20,w} = 2.5 \times 10^{-7}$ cm^2/s and $D_2(T = 20\ °C) = 0.48 \times 10^{-7}$ cm^2/s. Deviations of these values from the averages shown in Table I are indicative of the experimental error in an individual determination (see Experimental Section). Subtracting the exponential due to D_2 and plotting the resulting data semilogarithmically gives a straight line having a slope consistent with that expected from tubulin (dimer) and in agreement with the value of D^f reported in the text. The data quality of the semilogarithmic plot obtained after subtracting the exponential due to D_2 is poor and cannot exclude the presence multiple exponentials.

results support the earlier conclusion of Erickson (1974) that rings are in equilibrium with tubulin (dimer) and that pure tubulin (dimer) cannot be isolated using a sucrose gradient.

Discussion

The expected diffusion constant of tubulin (dimer) is estimated from the Svedberg equation (Tanford, 1961) to be 4.7×10^{-7} cm^2/s. Data presented in the Results section indicate that the tubulin samples prepared for our experiments contain more than one macromolecular component under all conditions examined. The quality of the correlation functions measured does not justify analysis in terms of more than two components. Thus it is possible that more than two components are present and that the diffusion constants reported are themselves averages over several components.

Sedimented Component between 10 S and 20 S. Comparison of the values of D^f for the HSX (<20S) solutions with D^f for the HSX (<10S) solutions clearly shows that D^f depends upon the degree of centrifugation. The diffusion coefficient expected for tubulin (dimer) is approached in the HSX (<10 S) solution. This implies that D^f measured for the HSX (<20 S) solution is an average diffusion constant of at least two components. Due to instrumental limitations, the multiple exponential decays which are expected were not resolved.

From our experiments we conclude that a component is preferentially removed in the HSX (<10S) preparations. The component is estimated to have $D_{20,w}$ (sedimented component) $\leq 3.2 \times 10^{-7}$ cm^2/s. The observation of a slow component having $\bar{D} \simeq 0.5 \times 10^{-7}$ cm^2/s means that the sedimented component must have $D_{20,w}$ greater than approximately 1.5×10^{-7} cm^2/s in order that it not be a separately resolvable component. Since D^f for the HSX (<10S) solution is experimentally that of a pure tubulin (dimer) solution, the sedimentation constant for the sedimented component must be between 10 S and 20 S.

Large Component. The diffusion constants for the large component found in the HSX (<20S) and HSX (<10S) preparations are the same within experimental error. The observation of the large component in the HSX (<10S) sample is particularly surprising in view of the extensive centrifugation to which the HSX (<10S) sample was subjected. It is possible that the large components observed in the HSX (<20S) and HSX (<10S) samples are different since the diffusion constant of a large structure is not very sensitive to details of the structure (Gethner, 1976).

Potential for Using Laser Light Scattering to Study Heterogeneous Protein Solutions. Laser light scattering has several advantages which should make it a valuable tool for investigations of heterogeneous protein systems.

(1) Solutions are probed at equilibrium under conditions where the analytical technique itself does not introduce strong perturbations.

(2) No physical separation of the components takes place. Thus a system fully capable of reacting or otherwise participating in a chemical equilibrium may be examined.

(3) The possibility of observing trace components exists since the scattering efficiency of a particle is roughly proportional to the square of the particle molecular weight. For particles which are different by as little as a factor of ten in molecular weight, the larger component is easily detectable in 1% concentrations (Gethner, 1976).

Acknowledgments

We would like to thank Professor C. R. Cantor for allowing us to use his facilities for some of the biochemical work.

References

Benedek, G. B. (1968), in Polarisation Matiere et Rayonnement, Paris, Presses Universitaire de France, pp 49-84.

Berne, B. J., and Pecora, R. (1974), *Annu. Rev. Phys. Chem. 25*, 233-253.

Berne, B. J., and Pecora, R. (1976), Dynamic Light Scattering, New York, N.Y., Wiley.

Chu, B. (1970), *Annu. Rev. Phys. Chem. 21*, 145-174.

Cummins, H. Z., and Swinney, H. L. (1970), *Prog. Opt. 8*, 133-200.

Doherty, P., and Benedek, G. B. (1974), *J. Chem. Phys. 61*, 5426-5434.

Erickson, H. P. (1974), *J. Supramol. Struct. 2*, 393-411.

Feit, H., Dutton, G. R., Barondes, S. H., and Shelanski, M. L. (1971), *J. Cell Biol. 51*, 138-147.

Gaskin, F., Cantor, C. R., and Shelanski, M. L. (1975), *Ann. N.Y. Acad. Sci. 253*, 133-146.

Gaskin, F., Kramer, S. B., Cantor, C. R., Adelstein, R., and Shelanski, M. L. (1974), *FEBS Lett. 40*, 281-286.

Gethner, J. S. (1976), Ph.D. Dissertation, Department of Chemistry, Columbia University.

Gethner, J. S., and Flynn, G. W. (1975), *Rev. Sci. Instrum. 46*, 586-591.

Gethner, J. S., Flynn, G. W., Berne, B. J., and Gaskin, F. (1977), *Biochemistry 16* (following paper in this issue).

Gorovsky, M., Carlson, D., and Rosenbaum, J. (1970), *Anal. Biochem. 35*, 359-370.

Hocker, L., Krupp, J., Benedek, G. B., and Vournakis, J. (1973), *Biopolymers 12*, 1677-1687.

Kirschner, M. W., Williams, R. C., Weingarten, M., and Gerbart, J. C. (1974), *Proc. Natl. Acad Sci. U. S. A. 71*, 1159-1163.

Koppel, D. E. (1972), *J. Chem. Phys. 57*, 4814-4820.

Koppel, D. E. (1973), Ph.D. Dissertation, Department of Physics, Columbia University.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265-275.
Pecora, R. (1972), *Annu. Rev. Biophys. Bioeng. 1*, 257-276.
Schaefer, D. W., and Berne, B. J. (1972), *Phys. Rev. Lett. 28*, 475-478.
Shelanski, M L., Gaskin, F., and Cantor, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70*, 765-768.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley.

Equilibrium Components of Tubulin Preparations†

Jon S. Gethner,‡ George W. Flynn, Bruce J. Berne, and Felicia Gaskin*

ABSTRACT: Laser light scattering techniques are used to examine the existence of a tubulin-ring or tubulin-aggregate equilibrium. Three principal components in addition to tubulin dimer have been observed. One large component having $D_{20,w} = 0.65 \pm 0.25 \times 10^{-7}$ cm^2/s is in equilibrium with tubulin (dimer) at 4 °C. A second large component having $D_{20,w} = 0.74 \pm 0.05 \times 10^{-7}$ cm^2/s is metastable at 4 °C and is formed when tubulin solutions are warmed to 36 °C and cooled back to 4 °C. The properties of the component reported in the previous paper (Gethner, J. S., Flynn, G. W., Berne, B. J., and Gaskin, F. (1977), *Biochemistry 16* (preceding paper in this issue)) to sediment at 10 S to 20 S are shown to be consistent with a small, metastable aggregate of tubulin.

The possibility that a tubulin-aggregate equilibrium may be present is suggested by the observation (reported in the preceding paper: Gethner et al., 1977a) of a large component having $D_{20,w} \sim 0.55 \times 10^{-7}$ cm^2/s in solutions which had been extensively centrifuged. In this paper we consider, in particular, the existence of a tubulin-ring or tubulin-aggregate equilibrium.[1] We have examined the equilibrium state of the system prior to temperature-induced assembly into microtubules and after cold-induced disassembly of the microtubules. Three components are described. Component A has $D_{20,w} \leq 3.2 \times 10^{-7}$ cm^2/s. Component B has $D_{20,w} = 0.65 \times 10^{-7}$ cm^2/s and is found before temperature-induced assembly. Component C has $D_{20,w} = 0.74 \times 10^{-7}$ cm^2/s and is found after temperature-induced assembly and disassembly. The possible identities of the various aggregates are discussed. Previously proposed mechanisms of assembly are evaluated in view of our results and limitations upon possible in vitro assembly mechanisms are discussed.

† From the Department of Chemistry, Columbia University, New York, New York 10027 (J.S.G., G.W.F., and B.J.B.), and the Departments of Pathology (Neuropathology) and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461 (F.G.). *Received February 2, 1977; revised manuscript received September 8, 1977.* Portions of this work have been presented at meetings of the American Physical Society (New York, February 1976) and the Biophysical Society (Seattle, February 1976). This work was taken, in part, from the Ph.D. Dissertation of Jon S. Gethner, Department of Chemistry, Columbia University, 1976. Support for the work was from grants from the National Science Foundation (NSF-CH-76-02414), the U.S. Public Health Service (NS-12418) and a Research Career Development Award to F.G.

‡ Present address: Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014.

[1] In this context, an aggregate specifically refers to a polymeric structure formed from tubulin (dimer) subunits. An aggregate may be a ring structure; however, no additional biochemical data are available to identify an aggregate as being a ring. A ring specifically refers to a polymeric structure observed in the light scattering experiments under conditions where rings have been shown to be present in substantial concentrations.

Experimental Section

Tubulin preparations and laser light scattering techniques are described in the previous paper (Gethner et al., 1977a). Three additional tubulin samples were used in this work.

(i) *LSX (<60 S)* samples were prepared as described previously for HSX (<20 S) samples except that the sample was centrifuged for 20 min.

(ii) *HSX (<20 S)-Mg* samples were prepared by adding $MgCl_2$ to a concentration of 5 mM after the protein was dialyzed. Centrifugation was performed at 4 °C following the procedures previously described for the preparation of a HSX (<20 S) sample.

(iii) *HSX (<20 S)-colchicine* samples were prepared by adding colchicine to a concentration of 100 μM to the dialysis buffer before beginning the dialysis. Dialysis was carried out at 4 °C in the dark. Except for the addition of colchicine, no modifications were made in the HSX (<20 S) preparation procedure. Two samples were prepared at each concentration. One was kept at 4 °C the entire time. The other was incubated at 26 °C for 30 min, conditions usually sufficient to result in approximately 0.2 mol of colchicine bound per mol of tubulin dimer (F. Gaskin, unpublished data). The sample was then cooled to 4 °C for 30 min before centrifuging.

Sedimentation velocity experiments were performed on a HSX (<20 S) sample containing only 0.1 mM GTP at 4 °C in a Spinco Model E ultracentrifuge operated at 40 000 rpm. Velocity profiles were scanned by measuring A_{280} for the sample contained in a double-sector cell. (Laser light scattering measurements on samples containing 0.1 mM GTP gave the same results as samples containing 1 mM GTP.)

Results

Components in Solutions Centrifuged to Remove 20 S

(1) *Analysis at 4 °C, 9 °C, and in the Presence of Colchicine at 4 °C.* Data is shown in Table I for a variety of samples prepared at 4 °C under conditions such that structures having $S \geq 20$ should not be present. $\bar{D}_N$ in Table I is the average diffusion constant ($\bar{D}$) for a two component mixture at 4 °C which