DYNAMIC LIGHT SCATTERING FROM SOLUTIONS OF MICROTUBULES

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ABSTRACT Calf brain microtubule protein was assembled in vitro to form dilute solutions of microtubules (240 Å diameter) having lengths >1 μm. The microtubule solutions were examined by dynamic laser light scattering techniques. The angular dependence of the correlation function leads to the conclusion that the correlation function is measuring the translational diffusion constant of the particles. The length dependence of the correlation function, however, shows that the translational diffusion constant is *not* being measured and that the diffusion constant for the microtubules cannot be straightforwardly determined. These results suggest that a collective property of the rods is being measured by the laser light scattering. Although specific microtubule-microtubule interactions are a possible explanation for the observed results, we present arguments that suggest that the solution can be adequately modeled as a network of entangled polymers.

INTRODUCTION

Dynamic light scattering techniques have recently been applied to the study of complex, multicomponent biopolymer systems. The systems examined generally fall into two classes as distinguished by the size of the polymers relative to the wavelength of the incident laser light (λ). One class includes particles with dimensions smaller than λ (see, for example, Gethner et al., 1977*a, b;* Cohen et al., 1975; Doherty and Benedek, 1974; Hocker et al., 1973). Aside from the problem of analyzing the correlation functions to obtain diffusion constants when multiple components are present, such systems can be treated with straightforward extensions of the theory developed for dilute homogeneous solutions (Berne and Pecora, 1976; Cummins and Swinney, 1970; Benedek, 1968). Another class is composed of long, filamentous structures greater than λ in length but smaller than λ in cross section (for example, Brenner et al., 1978; Carlson and Fraser, 1974; Tanaka et al., 1973). These latter systems, by virtue of the anisotropy of the molecules and the possibility, in some cases, of inter-

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molecular bonding, tend to form highly viscous solutions which are gels under some conditions. Analysis of such systems has been in terms of the viscoelastic properties of the gel network.

Microtubules, F-actin, hemoglobin-S polymers, and collagen are examples of the long, thin filamentous biopolymers which contain predominately one protein subunit and which can be assembled in vitro into polymers several microns in length. For dilute solutions of such polymers, it is tempting to extrapolate the theory applicable to heterogeneous solutions of small macromolecules and treat the long polymers as independently translating and rotating rods. The dynamic light scattering may then be used to measure the translational diffusion constant of the rod. Nonidealities such as the high microtubule concentration and the microtubule length will alter the interpretation of the line width measured. However, the line width, and hence an effective diffusion constant, will still measure the center-of-mass motion of the microtubule and thus be correlated to the length of the microtubules. We attempted such an analysis on microtubule solutions with the expectation that we could perform a detailed examination of both the length and length-distribution of microtubules. This would allow us to assess the validity of various mechanisms proposed for the assembly of microtubules. Electron microscopy was also used to estimate the lengths of microtubules.

MATERIALS AND METHODS

Laser Light Scattering Measurements

The spectrometer used for the light scattering measurements has been described previously (Gethner et al., 1977*a*; Gethner, 1976). All spectra were obtained by scattering light of 488 nm from a Spectra-Physics model 165 argon ion laser (Spectra-Physics Inc., Mountain View, Calif.) which was amplitude stabilized and operated on a single longitudinal mode. The output of the laser was attenuated with neutral density filters to between 0.05 and 0.3 mW. This level was sufficient to provide an optimum number of photon counts for the correlator used. The sample, contained in a sealed fluorescence cuvette, was at the center of a cylindrical water bath the temperature of which was maintained at 29 ± 0.2°C by a thermostatically regulated liquid circulator (Brinkmann Lauda K2R, Brinkmann Instruments, Inc., Westbury, N.Y.). A Channeltron photomultiplier (bialkali, Bendix Corp., Electro-optics Division, Southfield, Mich.) was used to provide a pulse input to a 3-bit counter and a Saicor 43-A digital autocorrelator. Autocorrelation functions obtained were analyzed on an IBM 360/91 computer system (IBM Corp., White Plains, N.Y.) using least squares regression techniques (Gethner, 1976). Correlation functions were normalized to the infinite time value of the correlation function by fitting the long time-points of the correlation function to a straight line by methods described previously (Gethner, 1977*a*).

To interpret the line widths measured, we assume that the scattering results principally from the center-of-mass motion of the microtubules. We may then interpret the experimental results by considering the dynamic light scattering from solutions of particles that are small relative to the wavelength of the scattered light. This has been extensively discussed both theoretically and experimentally (see, for example, Berne and Pecora, 1976; Cummins and Swinney, 1970; Benedek, 1968). For a solution of noninteracting Brownian particles, which are small relative to the wavelength of light, the homodyne intensity autocorrelation function is given by $C(t) = \langle I(0)I(t)\rangle = \langle I(t)\rangle^2[1 + (1/N)\,|\,e^{-\Gamma t}\,|^2]$. $I(t)$ is the intensity of light observed at the time, t. The brackets, $\langle\ \rangle$, indicate time averaging and $\Gamma = Dk^2$.

k is the magnitude of the scattering vector $= (4\pi n/\lambda)\sin(\theta/2)$, with λ the wavelength of the incident light, n the solution index of refraction, and θ, the scattering angle. D is the translational diffusion constant of the particle, and N is an apparatus constant. If the solution is either polydisperse or heterogeneous, the form of $C(t)$ remains unchanged though Γ (and thus D) becomes an average over the components in the solution (Koppel, 1972; Gethner et al., 1977*a*). Γ obtained at several scattering angles and plotted against k^2 will lie on a straight line having a zero intercept. The slope of the line will just be D (or the weighted value). A knowledge of D obtained either from the slope of Γ vs. k^2 or by analysis of Γ taken at a single scattering angle allows the hydrodynamic radius of the particle to be calculated (Tanford, 1961).

$D = k_b T/6\pi\eta r$, where k_b is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and r is the hydrodynamic radius of the particle. Thus it is clear that for a series of particles of increasing radius, Γ must decrease.

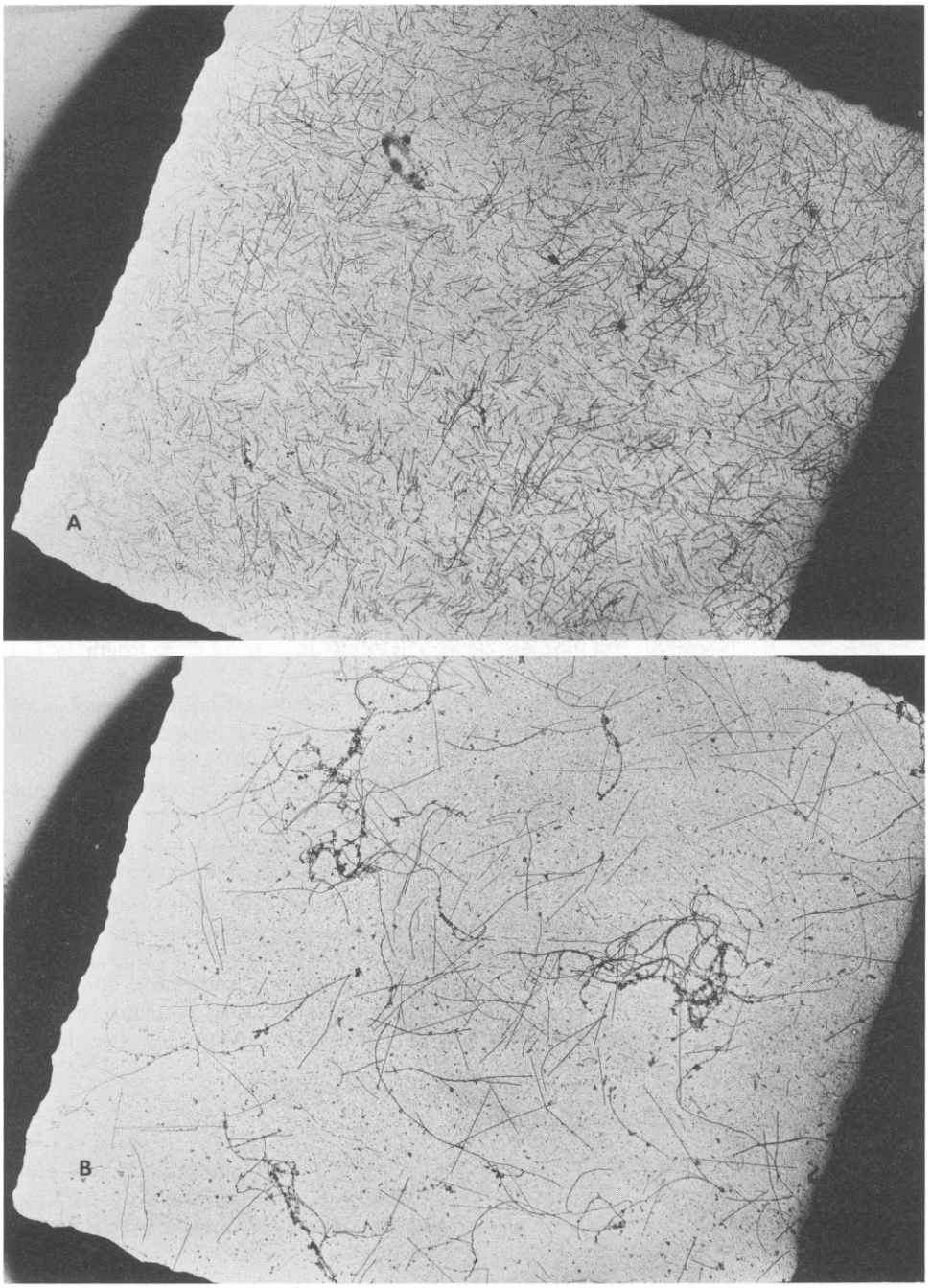
For many solution nonidealities, the center-of-mass motion may be retarded, and Γ obtained from such solutions cannot be straightforwardly related to the translational diffusion constant of the particle. Nevertheless, we expect the same qualitative feature as is predicted by considering the ideal solution when the line width is plotted against either the length of the particle or k^2. In fact, Lee et al. (1977) have considered the scattering from a solution of large, semiflexible macromolecules in concentrated solutions and find a component in the spectrum due to the center-of-mass motion which exhibits k^2 scaling and scales with size as does the ideal particle. They find nonzero intercepts in plots of Γ against k^2 due to the coupling of internal motions with the translational center-of-mass motion. Such nonzero intercepts have been found by other workers (Fujimi, et al., 1971) and have been cited as evidence of intramolecular motion. The fact that the line width is still simply related to the macromolecular size in the highly nonideal solutions considered by Lee et al. (1977) leads us to expect that the line width will also be simply related to the microtubule length in our experiments.

Correlation functions obtained from our solutions of microtubules are not well described by a single exponential decay and the normalized second cumulant is 0.5–1.0 for a second order fit. Semilogarithmic plots of the normalized correlation functions are curved over the entire time range of data collected. We have analyzed the correlations functions by carrying out the cumulant fit through 4th order and additionally have observed that the correlation functions may be parameterized adequately by a fit to the sum of two exponential decays. In the absence of a theory to relate the experimentally determined line width to the measurement of a physical property, there is no a priori reason for choosing a particular fitting scheme. We have chosen to present the data obtained by 2nd order fits. All correlation functions were analyzed by both a single exponential fit and a 3rd and 4th order cumulant fit. The qualitative features of data presented in the figures are unaltered if we use these alternative fitting procedures. Multiple exponential fitting of the data also results in the same qualitative features shown in the figures. In particular, k^2 scaling is found for both exponentials, and the intercept of a k^2 plot is zero.

Preparation of Microtubule Protein

Calf brain microtubule protein was purified from fresh brains after the recyclization procedure developed by Shelanski et al. (1973). Microtubule protein which had been stored at −20°C in 4 M glycerol in buffer (0.1 M 2-[*N*-morpholino] ethanesulfonic acid (MES)[1] pH = 6.55, and con-

[1] *Abbreviations used in this paper:* EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; MAPs, microtubule associated proteins; MES, 2-(*N*-morpholino)-ethanesulfonic acid; SDS, sodium dodecyl sulfate.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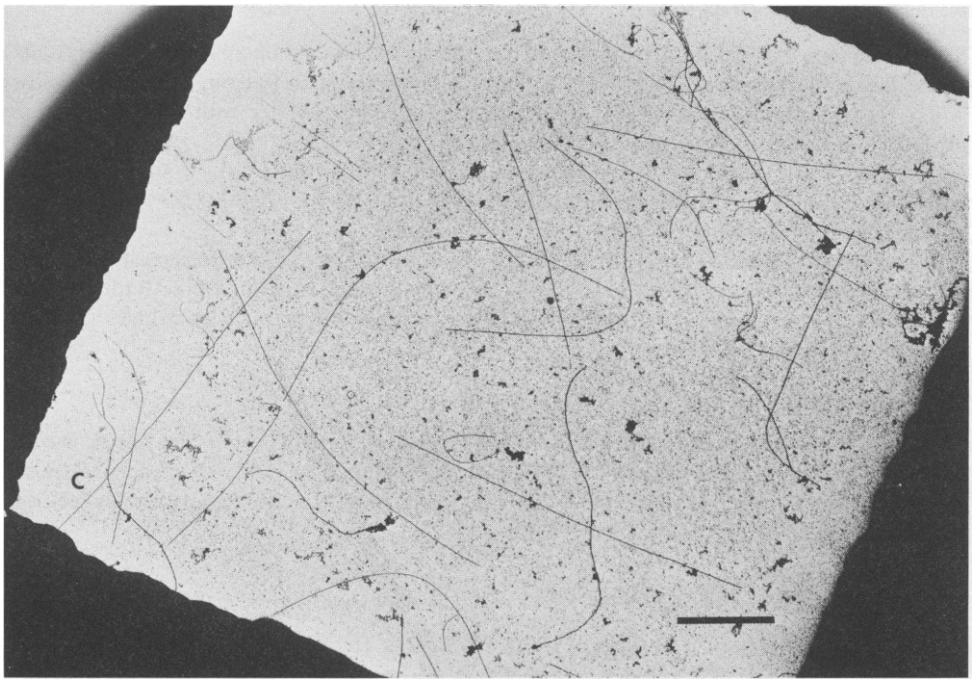

FIGURE 1 Electron micrograph demonstrating an increase in the average microtubule length (L) as the initial microtubule protein concentration (C_o) decreases. Scale bar equals 10 μm. (a) C_o = 3.3 mg/ml. L = 3.3 μm. Protein concentration (MT) = 2.2 mg/ml (based upon a Lowry determination of protein in a pellet after centrifugation). (b) C_o = 0.55 mg/ml. L = 8.7 μm. (MT) = 0.21 mg/ml. (c) C_o = 0.33 mg/ml. L = 30 μm. (MT) = 0.05 mg/ml.

taining 10^{-3} M ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) and 5×10^{-4} M $MgCl_2$) was dialyzed for 3 h at 4°C against 250 vol of buffer. Guanosine 5′-triphosphate (GTP) to 1 mM was added. 3.5 ml of tubulin was centrifuged at 4°C for 30 min using a Beckman type 65 rotor (Beckman Instruments, Inc., Fullerton, Calif. operated at 35,000 rpm. Approximately 0.5 ml of the sample was withdrawn from the centrifuge tube using a precooled 1-cm^3 disposable syringe, transferred into a precooled fluorescence cell and sealed with a Teflon stopper. Aliquots of this solution were diluted with centrifuged buffer. The samples were assembled at 36°C for at least ½ h and stored at 29°C until correlation functions were run. The protein concentration of the most concentrated solution was determined by the method of Lowry et al. (1951), and the dilution factor was used to calculate the concentrations of the remaining samples.

Polyacrylamide Gel Electrophoresis

Microtubule protein preparations were analyzed in 5 and 7% polyacrylamide gels using the discontinuous sodium dodecyl sulfate (SDS) urea system (Feit et al., 1971; Shelanski, 1974) as previously described for similar protein preparations (Gaskin et al., 1974; Gaskin et al., 1975). As expected, the preparation contained approximately 85% tubulin and 15% microtubule associated proteins (MAPs). The major MAPs had apparent molecular weights of 370,000 and 355,000 as previously reported (Gaskin et al., 1974).

Electron Microscopy

Samples examined by light scattering were also examined by electron microscopy. In procedure 1, one drop of the sample was applied to a Formvar grid (Ernest F. Fullam, Inc., Schenectady, N.Y.), rinsed with six drops of 1% uranyl acetate, and blotted dry with filter paper. In procedure 2, microtubules were fixed by adding an equal volume of 2% glutaraldehyde in MES buffer. After 15 min at 30°C, a drop was placed on carbon-over-Formvar glow discharged grids and treated as previously described by Sloboda et al., (1976) using 0.2% cytochrome-c in 1% amyl alcohol for rinsing and 1% uranyl acetate for staining.

A Siemens 102 (Siemens Corp., Iselin, N.Y.) was used for the electron microscopy. At least 100 microtubules were measured in a field on two grids at each concentration. Both methods of preparation gave similar results, although the average length of microtubules fixed in glutaraldehyde was 7% longer.

Materials

2-(*N*-morpholino)ethanesulfonic acid was Calbiochem grade A (Calbiochem, San Diego, Calif.). GTP (type II-S) was from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were standard reagent grade. Distilled water used for cleaning cells was filtered through 0.22-μm Millipore filters (Millipore Corp., Bedford, Mass.).

RESULTS AND DISCUSSION

An analysis of electron micrographs shows that as the initial concentration of the microtubule protein increases, the average microtubule length decreases (see Fig. 1). Similar results have been previously reported (Engelborghs, et al., 1977).

Table I summarizes the physical parameters of a series of microtubule solutions prepared from different initial concentrations of protein at 29°C. The light scattering

TABLE I

DEPENDENCE OF THE AVERAGE LENGTH (L) OF MICROTUBULES AND OF V_R/V* ON THE INITIAL CONCENTRATION C_O IN A TYPICAL EXPERIMENT

C_o	L‡	V_r/V
mg/ml	*μm*	
4.95	6.0 ± 2.8	239
1.14	8.9 ± 4.1	119
0.71	11.3 ± 6.3	119
0.55	14.8 ± 8.5	158
0.45	16.0 ± 6.5	152
0.35	27.3 ± 9.0	349
0.29	27.3 ± 13.6	247
0.24	≥37.9 ± 17.7§	≥449

*V_r = the total volume of the rotating rods of a given length, and V is the total volume. (See Results and Discussion.)

‡Error is 1 SD assuming the distribution to be a Gaussian.

§Length is a lower limit due to the inclusion of tubes that were partially outside the observation grid. The average length calculated using only those tubes contained within the grid is 38.4 ± 17.7 μm.

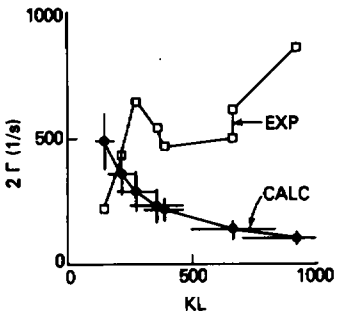

FIGURE 2

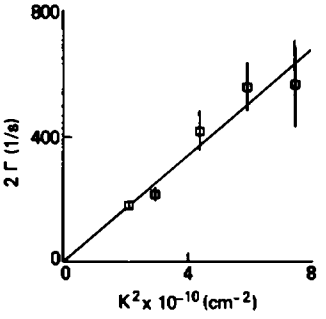

FIGURE 3

FIGURE 2 Length dependence of the line width of microtubule solutions $\theta = 90°$, $k = 2.4 \times 10^5$ cm^{-1}. The experimentally obtained points ([□] connected by a solid line) have errors comparable to the size of the points as judged by 1 SD of the mean for a set of measurements performed on the same sample. Points (○) were calculated by assuming the microtubule to be a prolate ellipsoid (see Tanford, 1961) having a diameter of 240 Å and the length shown in Table I for the appropriate solution. The error bars shown for the calculated points represent the width of the distribution of the lengths (see Table I) and the corresponding uncertainty introduced into the calculation of 2 Γ.

FIGURE 3 Line width of the sample having $L = 8.9 \pm 4.1$ μm ($C_o = 1.14$ mg/ml) as a function of the square of the scattering vector (k^2). The line drawn is the result of a linear least squares fit to a straight line which has been constrained to have a zero intercept. Fitting the intercept does not result in a significant change in the slope, and the value obtained for the intercept is zero, to within experimental error. The error bars are 1 SD of the mean for a set of measurements performed on the same sample.

results of the same solutions at $\theta = 90°$C are shown in Fig. 2. The line width, Γ, is proportional to Dk^2, where D, in this case, is understood to mean an "effective" diffusion constant. If the microtubule solution could be described as a solution of translating rigid rods, we expect Γ to be inversely proportional to the length of the microtubules. This is clearly not the case for the solutions shown in Fig. 2. In fact, over much of the length range shown, the qualitative behavior indicates that Γ is directly proportional to length. Inclusion of solution nonidealities and even resorting to a fairly complete description of semiflexible macromolecules undergoing hindered translation (Lee et al., 1977) does not account for the qualitative trend shown in the figure (see Light Scattering Measurements above).

In Fig. 3 we show the results of the angular dependence of the line width plotted vs. k^2 for the solution having $C_o = 1.14$ mg/ml (see Table I). It can be seen that within experimental error, the line width scales with k^2 and has a zero intercept. These results are what is expected for a solution of Brownian particles. The slope of the line gives an estimate for the "effective" diffusion constant of $D_{20,w} = 0.33 \times 10^{-8}$ cm^2/s. The apparent k^2 scaling of D shown in the figure leads one to believe that the light scattering can be treated adequately by theory which is applicable to noninteracting Brownian particles. The clearly improper scaling with length shown in Fig. 2, however, indicates that the line width is not merely measuring the translation of the microtubules through the solution.

Two ways to explain the laser light scattering results include (*a*) the existence of specific microtubule-microtubule interactions, and (*b*) the formation of a network of loosely entangled polymer strands. We will discuss these possibilities separately.

Specific Microtubule-Microtubule Interactions

Examination of grids containing microtubules prepared as described in Materials and Methods and illustrated in Fig. 1 does not reveal any obvious microtubule-microtubule interactions. However, our microtubule preparations contain approximately 85% tubulin and 15% MAPs. Both electron microscopy and viscosity studies have been used to support specific microtubule-microtubule interactions in such preparations. Thin-section electron microscopy of microtubules pelleted through discontinuous sucrose-glycerol gradients demonstrated "bridging" between microtubules as well as "arms" (Gaskin et al., 1974). The "arms" are present on microtubules polymerized in the presence of MAPs (Dentler et al., 1975; Murphy and Borisy, 1975). The viscosity of microtubule solutions in the presence of MAPs is much higher than in the absence of MAPs or after trypsin cleavage of the MAPs (Griffith and Pollard, 1978). Clearly laser light scattering studies and length determinations should be performed on microtubules assembled in the absence of MAPs and on microtubules containing MAPs selectively removed by trypsin. The results of such a study might be a more definitive test of specific microtubule-microtubule interactions mediated by MAPs. Such a study has the further advantage of being able to be performed at low protein concentration.

Entangled Polymer Network

Interactions may exist between the microtubules by virtue of the fact that the very long, rodlike molecules may be in sufficient concentration that free diffusion of the microtubules is prevented. Assuming each microtubule to be a rigid rotor, we can calculate the ratio of the total volume swept out by the rods (treated as freely rotating rigid rods) and the actual solution volume, A rough estimate of the rod concentration at which interactions *cannot* be ignored is the number of rods giving $V_r/V \gtrsim 1$; V_r = the total volume swept out by the rotating rods of a given length, and V is the total volume. $V_r/V \gg 1$ for all the microtubule solutions studied (Table I).

The concentrations of microtubules employed in these experiments are sufficiently low that our solutions cannot be modeled as a gel. The ratio $V_r/V \gg 1$ suggests that the density of microtubules may be sufficiently large that our results may be interpreted in terms of the theory formulated by de Gennes (1976*a*, *b*) for entangled polymers. Though the microtubules are not strictly the same physical system as described by de Gennes, the motivation for comparing our experimental results to the scaling laws of de Gennes is as follows. At sufficiently high concentration, a solution of long rods will not behave as a solution of independently translating and rotating particles because the volumes swept out by each rotating rod must intersect with neighboring rods and there will be a high probability that two rods will collide. Nevertheless, if the collision frequency is low, it seems reasonable that the only effect of the collisions will be to retard, but not greatly restrict, the motion of the rod relative to its free translation and rotation motion. Because the volume fraction of micro-

tubules is very small, our system is probably accurately described as independently translating rigid rods which collide with a low, but not negligible, probability. Although the theory of de Gennes (1976*a*, *b*) is for a flexible polymer system, it is expected that de Gennes' results will be applicable to a range of polymers from freely jointed chains to stiff rods because the theory considers the problem of how to move a polymer through the solution when there are "points of entanglement." Collisions between rigid rods can be viewed as effectively generating points of entanglement. The relative stiffness of a polymer would only result in a rescaling of the mean free length between entanglement points.

For a solution of polymer chains, the line width, $\Delta\omega_k$, is given by (de Gennes, 1976*b*):

$$\Delta\omega_k = \Delta f_m(k\xi). \tag{1}$$

$f_m(x)$ is a dimensionless function describing the scattering as a function of the magnitude of the wave vector, k, and a correlation length, ξ, describing the distance between entanglements. Δ is a characteristic frequency given by $\Delta = k_b T/6\pi\eta\xi^3$. $f_m(x)$ has the following limits:

(*a*) For $x \to 0$, $f_m(x) \to x^2$. This case applies when the mean distance between entanglements is $\ll 1/k$—the polymers must be contacting each other with very high frequency.

(*b*) For $x \gg 1$, $f_m(x) \to x^3$. This case applies when the distance between entanglements is large and the contact frequency is small. However, it should be noted that extrapolation of this case to zero polymer concentration cannot be correct for infinitely stiff rods because the result should recover the case of independently translating and rotating rods for which D scales as k^2.

Depending upon whether a or b applies, a plot of Γ vs. $(kL)^2$ or $(kL)^3$ will give a straight line. It should be noted that the intercept of such a plot need not be zero because both the $(kL)^2$ and the $(kL)^3$ dependence are derived for particular limiting conditions.

In Fig. 4 we have replotted the data of Fig. 3 vs. the dimensionless parameter $(kL)^3$. We expect L to be proportional to ξ (though not necessarily in a linear fashion). The data plotted in Fig. 4 can be adequately fit by a straight line. The scatter of the data points in both Fig. 3 and 4 does not allow an unambiguous determination of $(kL)^2$ or $(kL)^3$ scaling. However, assuming $(kL)^3$ scaling, the mean distance between entanglements can be estimated from Eq. 1 to be 5 μm. This distance suggests that the rods behave as a very loose network of entangled strands.

Further investigation must be able to independently vary both the number concentration and the lengths of the rods. Investigations using a narrow length distribution and varying both the number concentration and lengths of the rods could clarify the results from this system. Especially interesting would be the transition region $kL = 1$.

It is clear, also, that a transition to single particle behavior should be observable even at moderate concentrations if the particles are sufficiently short. Preliminary data on microtubules, in fact, show such a transition when Γ vs. L is examined. Our inability to conveniently prepare microtubule solutions of a specified length and number concentration has hampered such investigations.

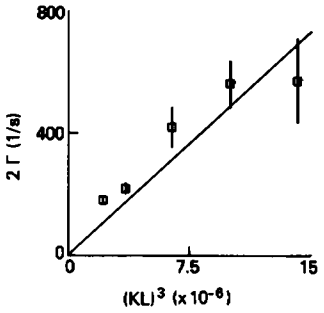

FIGURE 4 Line width of the sample having $L = 8.9 \pm 4.1$ μm (C_o = 1.14 mg/ml) as a function of (kL).[3] The straight line drawn is the result of a linear least squares fit as described for Fig. 3. Error bars are as in Fig. 3.

If the scaling properties of de Gennes (1976*b*) apply to these solutions, accurate angular measurements at very large k would be desirable. This can best be achieved using short wavelength light such as from a UV laser.

SUMMARY

The principle result obtained from our study is that the reciprocal of the effective diffusion constant does not necessarily scale with the length of the microtubules as would be expected if the solution could simply be treated as a multicomponent solution containing microtubules undergoing translational diffusion. No satisfactory theory currently exists with which to describe our system. Microtubule-microtubule interactions are a possible explanation. We have argued, however, that our physical intuition of the properties of microtubule solutions suggests that the recent work of de Gennes (1976*a*, *b*) on the dynamics of entangled polymers may apply to such solutions. It is important to note that the observation of inconsistencies such as we find for microtubule solutions could be observed in any similar polymer solution. Experiments employing laser light scattering to look at the mechanism of self-assembly systems may not be measuring a diffusion constant, and although the line width can still be expected to be correlated with the particle size, the interpretation of the experiments may be different than if the principal component of the spectrum is due to translational diffusion.

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