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Transient electric birefringence study of rod-like triple-helical polysaccharide schizophyllan

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The rotational dynamics of the uncharged, triple-helical polysaccharide schizophyllan was determined by transient electric birefingence. The field-free decay of a sample with weight average molecular weight of 437×10^3 gmol^{-1} deviated from single-exponential behaviour. The relaxation spectra obtained by analysis using the CONTIN program, were bimodal, whereas analysis by the stretched exponentials also accounted well for the field-free decay of the birefringence of the polydisperse sample. The mean rotational relaxation time extrapolated to infinite pulse duration of the orienting pulse for dilute aqueous solution of the 437×10^3 gmol^{-1} sample was determined equal to 61 μs. Analysis of the influence of chain rigidity on the rotational dynamics of the polydisperse sample indicated that the persistence length, L_{pe}, was 70nm. Copyright © 1996 Elsevier Science Limited.

INTRODUCTION

Schizophyllan is a polysaccharide extracted from the mycelium of the fungi *Schizophyllum commune*. The purified polysaccharide dissolves readily in aqueous solution yielding a large viscosity increase of the solution unless the sample extraction and purification include depolymerization steps. This polysaccharide is commercially available as a partly physically depolymerized sample (Misaki *et al.*, 1993), and is used in this high-molecular weight form as a broad immuno-stimulating agent (Chihara, 1984; Misaki *et al.*, 1993). The latter studies have reached the stage of clinical applications for the treatment of certain types of cancers in Japan (Okamura *et al.*, 1986). The function as a biological response modifier (BRM) is closely connected to the chemical structure of schizophyllan, i.e., the comb-like branched tetrasaccharide repeating unit of (1→3)-β-D-Glcp with a 1→6 linked β-D-Glcp sidechain (Chihara, 1984; Misaki *et al.*, 1993). The BRM action is also reported to depend on the triple-helical structure using inhibition of sarcoma S180 implanted in mice as the test system (Yanaki *et al.*, 1983). Chemically analogous naturally occurring glucans, such as lentinan and scleroglucan, have also been reported to show similar immune-stimulating activity (e.g. Chihara, 1984; Pretus *et al.*, 1991; Misaki *et al.*, 1993). Increased interest has recently also emerged in the western world towards this family of polysaccharides, not only for their action as BRMs in mammals, but also for use as adjuvants in vaccination of fish (Matsuyama *et al.*, 1992; Nikl *et al.*, 1993).

The action of schizophyllan as a BRM is closely connected to the triple-helical structure adopted in solution. The hydrodynamic properties of this polysaccharide have been determined experimentally using viscometry, light scattering, and high-frequency dilute solution rheology. In this study, the hydrodynamic characterization of schizophyllan is extended by determination of the rotational diffusion constant (D_r), or an apparent rotational relaxation time (τ_{rel}) employing transient electric induced birefringence (TEB). The parameter D_r is determined from the decay of the birefringence after abrupt removal of the electric field orienting the molecules. In the rigid rod-like limit, D_r shows an inverse third power dependence on the axial ratio (Broersma, 1960). This suggests a large sensitivity towards differences in contour length, or molecular weight, within a series of homologous samples. The TEB technique has found widespread use in studying the rotational dynamics of polynucleotides (Hagerman, 1981; Stellwagen, 1981) and elongated proteins (e.g. Kobayasi & Totsuka, 1975; Highsmith *et al.*, 1977; Mikkelsen & Elgsaeter, 1978), but is in general applied less to the study of polysaccharides (Foweraker & Jennings, 1977; Trimm & Jennings, 1983; Besio *et al.*, 1987; Stellwagen & Stellwagen, 1990). Both the inherent flexibility of many polysaccharides as well as the diffi-

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culty in obtaining polysaccharide samples with reduced polydispersity are among the reasons why polysaccharide samples are more difficult to study than the members of the other two main classes of biopolymers.

The study of rotational dynamics of a homologous series of hydroxyethyl cellulose reported by Foweraker & Jennings (1977) illustrates the difficulties in the interpretation of data obtained from TEB associated with chain flexibility. These authors report an increase in an average τ_{rel} with increasing weight average molecular weights (M_w) for M_w below 40×10^3gmol^{-1}. Further increase in M_w did not yield the expected correlation between the mean rotational relaxation time and M_w, instead they observed that τ_{rel} levelled off at a constant value independent of M_w. Foweraker and Jennings suggested that this could be because the dominating orientational mode contributing to the birefringence was sub-molecular segments reorientation and not the whole polymers as such. The optical signal thus probed internal relaxation processes and not the overall rotational relaxation. The TEB technique has, on the other hand, proved to be very sensitive to chain flexibility close to the rod-like limit, and this subject has been pursued in a number of experimental works on DNA (Hagerman, 1981; Stellwagen, 1981; Lewis *et al.*, 1986) and proteins (Highsmith *et al.*, 1977; Mikkelsen *et al.*, 1985) as well as theoretical studies (Yoshizaki & Yamakawa, 1980; Hagerman & Zimm, 1981). The persistence length can be estimated by comparison of τ_{rel} with that calculated in the rigid rod-like limit for the polymer under investigation. Using this approach, Hagerman (1981) reported that the persistence length of DNA was 50nm for salt concentrations larger than 1mM. The electrostatic contributions to the persistence length of DNA at these conditions are negligible compared to the structural rigidity.

The other limiting factor in applying the TEB technique to studying rotational dynamics of polysaccharides arises from the chain length heterogeneity of the samples. Proteins and deoxyribonucleic acids are obtainable as monodisperse samples, and the analysis of the birefringence decay is in practice limited to fitting the observed decay to a model valid for such an idealized case. But even for the monodisperse case, relaxation modes arising from segmental motion of the molecule are expected to add to the overall decay of the birefringence from the sample (Lewis *et al.*, 1986). How well internal modes are separated from the τ_{rel} for the overall rotation depends on chain flexibility (e.g. Ferry, 1980). In a polydisperse sample, each of the components is expected to contribute to the birefringence decay with a weight determined by its relative weight fraction if its relaxation mode has been excited. This means that for polysaccharide samples with reduced polydispersity, i.e., with the ratio between the weight and number average molecular weight M_w and M_n in the region of 1.5, one may expect relaxation times for internal modes to be overlapping the overall relaxation times of the smaller components.

MATERIALS AND METHODS

Samples

Schizophyllan samples U-1 and M-2 were kindly provided as freeze-dried material from Taito Co., Kobe, Japan. The high molecular weight of the polysaccharide originally isolated from the mycelium in the fermentation broth, had been depolymerized using ultrasound irradiation to lower the molecular weights (Misaki *et al.*, 1993). Samples U-1 and M-2 have been extensively characterized with respect to static properties in aqueous solution, and reported previously (Table 1). The samples were dissolved in Milli-Q water by stirring overnight. The schizophyllan solutions were centrifuged at $18\,000\times g$ (15 000rpm, Beckmann 50Ti rotor) at $T=20$°C for 60min, before the electric birefringence measurements. No visible pellet was observed after centrifugation. The polysaccharide concentrations were determined as the ratio between the weighted amount of freeze-dried sample to the volume of Milli-Q water in which it was dissolved.

Transient electric birefringence—experimental setup

The transient electric birefringence was measured using previously described instrumentation (Mikkelsen & Elgsaeter, 1978, 1981). A quarter-wave plate analyzer was positioned between the Kerr cell and the analyzer, and the optical axis was adjusted until the effect of strain birefringence in the Kerr cell was minimized. The

Table 1. Physical properties of schizophyllan samples U-1 and M-2[a]

Method	Parameter	U-1	M-2
Gel permeation chromatography	M_n, g mol^{-1}		334×10^3
Light scattering	M_w, g mol^{-1}	134×10^3	437×10^3
Viscometry	$[\eta]$, ml g^{-1}	58.4	404
Electron microscopy	L_n, nm	59	164
Electron microscopy	L_w, nm	65	210

[a]From Stokke *et al.* (1993).

electrical excitation pulse of this system showed a decay time of 0.15±0.05μs. With propylene carbonate in the Kerr cell and using the quadratic detection mode, the decay time of the optical pulse was measured to be 0.09±0.02μs. Propylene carbonate exhibits an extremely rapid birefringence relaxation and therefore is appropriate for a test of the transient response of the system.

The duration of the excitation pulse, t_{pulse}, could be adjusted up to about 1ms and the pulse height up to about 1.5kV across the electrodes, which are positioned 2.0mm apart. These maximum values depend generally on the salt concentration of the sample solution.

The electrical excitation pulse and the optical pulse were recorded using a Philips 125MHz digital storage oscilloscope type PM 3315. The data were transferred to a standard IBM compatible computer using IEEE interface and GPIB software.

Analysis of birefringence decay

The birefringence decay, $\Delta n(t)$, of a polydisperse ensemble of polymers can be written as:

$$\Delta n(t) = \sum_{i=1}^{N} \Delta n_i^0 e^{-t/\tau_i} \quad (1)$$

where Δn_i^0 is the contribution to the total birefringence from exponential component i, τ_i is its associated relaxation time, and N is the number of components in the ensemble. By identification of component i with a given chain length, Δn_i^0 should be taken to reflect both the fraction of chains with this length as well as possible orientation functions corresponding to this decay time. Analysis of multicomponent decays to obtain relaxation spectra without additional assumptions is mathematically ill-conditioned and therefore not a straightforward procedure. The data analysis program CONTIN (Provencher, 1982a, b) chooses the distribution of decay times that best fit the data using the principle of parsimony. Or in other words, the distribution having the least number of parameters is preferred compared to a larger set of parameters. The data analysis using CONTIN will be carried out without any further presentation of the method. The application of this analysis approach was evaluated by analyzing computed composite decay curves to which various noise-levels were added.

Recently, relaxations in polydisperse systems have been described in terms of a normalized stretched exponential function, $R(t)$ (Bellini *et al.*, 1989; Dormoy & Candau, 1991):

$$R(t) = \frac{\Delta n(t)}{\Delta n(t=0)} = \exp(-(t/\tau_{rel})^{\beta}) \quad (2)$$

where $\Delta n(t=0)$ is the birefringence just after the orienting pulse is switched off, τ_{rel} is a relaxation time, and β a relaxation exponent. The β-exponent depends on the distribution of chain lengths and the function that weights the contribution of each chain length to the electro-optic signal. The average relaxation time, $<\tau_{rel}>$, is calculated as (Dormoy & Candau, 1991):

$$<\tau_{rel}> = \tau_{rel}\Gamma(1/\beta)/\beta \quad (3)$$

where Γ is the gamma-function.

The stretched exponential analysis was carried out both on the experimental data, and on composite relaxation curves calculated based on the observed contour length distribution of M-2 using electron microscopic methods (Stokke *et al.*, 1993). The composite relaxation curves were calculated assuming complete orientation of all species and the rotational relaxation time for a given contour length was calculated assuming a rigid cylinder using the Broersma equation, and for persistence lengths, L_{pe} from 50 to 200nm using the interpolation formulae provided by Yoshizaki & Yamakawa (1980). The composite relaxation of all species in the experimentally determined contour length distribution was then calculated (eqn (1)), which subsequently was subjected to the analysis employing the stretched exponential (eqn (2)). This procedure yielded predicted average relaxation times (eqn (3)) that depended on the persistence length, and could therefore be used as a basis for determination of L_{pe} from the $<\tau_{rel}>$ determined from the experimental data.

RESULTS AND DISCUSSION

Figure 1 shows examples of birefringence raw data for schizophyllan M-2 at a concentration of 0.5mg/ml in Milli-Q water. The data examples were obtained using excitation field strength of $E=0.4\text{kVnm}^{-1}$ and pulse duration of $t_{pulse}=0.6$ms and $E=0.6\text{kVmm}^{-1}$, $t_{pulse}=0.15$ms, in Fig. 1(a) and (b), respectively. The data suggest that the decay is faster for the shortest excitation pulse, indicating that the longest polysaccharide chains are not fully oriented. Increasing the duration of the excitation pulse yields a decay that contains relatively more of the slower decaying processes.

Figure 2 shows examples of relaxation spectra obtained using the CONTIN program on the data depicted in Fig. 1. Both relaxation time spectra are apparently bimodal. The 0.6kVmm^{-1}, $t_{pulse}=0.15$ms excitation yields a spectrum (Fig. 2(a)) with the major maxima centered at $\tau_{rel}=59\mu$s, and a minor peak at $\tau_{rel}=12\mu$s. A third very small component at $\tau_{rel}=2\mu$s was also observed. For $E=0.4\text{kVmm}^{-1}$, $t_{pulse}=0.60$ms, the major peak is shifted towards longer relaxation time and is centered around $\tau_{rel}=100\mu$s. The minor peak is located around $\tau_{rel}=14\mu$s (Fig. 2(b)). The intensity ratios between the two peaks in the spectra were nearly unchanged. The longest relaxation times in these spectra were observed to shift from about $\tau_{rel}=100\mu$s to $\tau_{rel}=250\mu$s in response to the changes in the excitation pulses.

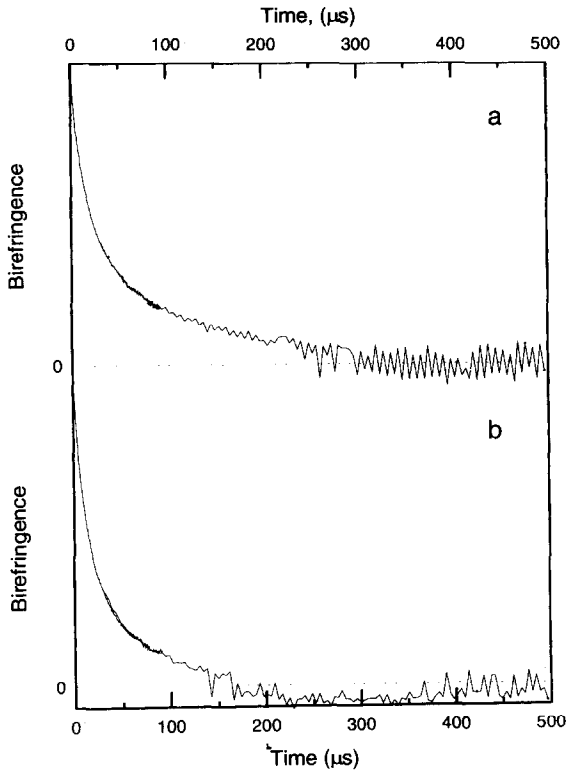

Fig. 1. Birefringence decay of 0.5 mg/ml schizophyllan M-2 after orienting using (a) 0.4 kV mm^{-1} and pulse duration 0.6 ms, and (b) 0.6 kV mm^{-1} and pulse duration 0.15 ms.

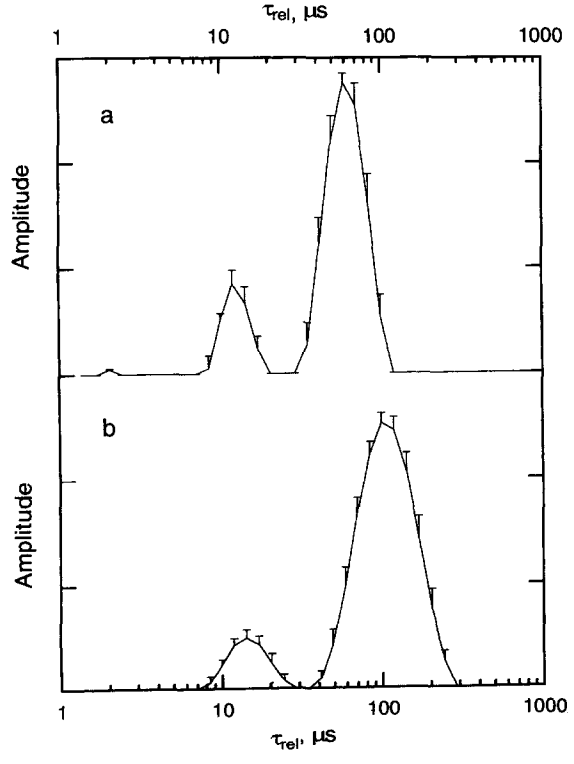

Fig. 2. Decay time spectra calculated using the CONTIN program on the birefringence decay data of 0.5 mg/ml schizophyllan M-2 using excitation pulse of (a) 0.4 kV mm^{-1} and $t_{pulse}=0.6$ ms, and (b) 0.6 kV mm^{-1} and $t_{pulse}=0.15$ ms.

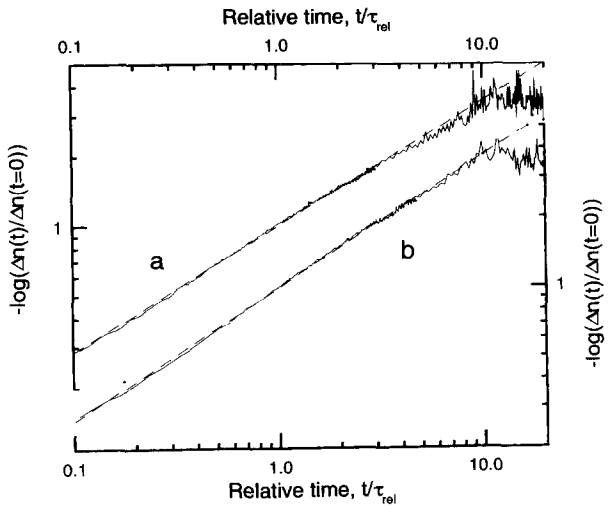

Fig. 3. Normalized decay functions R versus reduced time for the electrically induced birefringence of 0.5 mg/ml schizophyllan M-2 using excitation pulse electric field strength E of (a) $E=0.4$ kV mm^{-1} and pulse length $t_{pulse}=0.6$ ms, and (b) $E=0.6$ kV mm^{-1} and $t_{pulse}=0.15$ ms.

Figure 3 shows that the stretched exponential function for both conditions shown can represent the decay data for times ranging two orders of magnitude centered around τ_{rel}. This was found as a general trend for all the data. The deviation observed for $t/\tau_{rel} > 10$ is most likely due to the poor signal-to-noise ratio for the small signals. The relaxation times and exponents β for the given examples were determined to be $\tau_{rel}=22.2\mu s$, $\beta=0.631$, and $\tau_{rel}=36.5\mu s$, $\beta=0.595$ for the excitation pulses employed in Fig. 3(a) and (b), respectively. The average values within the distribution calculated according to eqn (3), were found to be $<\tau_{rel}>=31.4\mu s$ and $<\tau_{rel}>=55.5\mu s$, respectively. These average relaxation times are on the short time side of the major peak in both the relaxation spectra obtained using the CONTIN analysis (Fig. 2).

Figure 4 depicts $<\tau_{rel}>$ obtained using the stretched

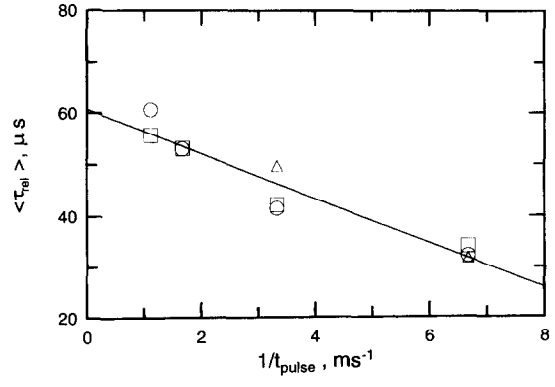

Fig. 4. Average relaxation times $<\tau_{rel}>$ obtained using stretched exponential analysis versus the inverse of pulse length for 0.5 mg ml^{-1} schizophyllan M-2. The symbols denote excitation field strengths of 0.2 kV mm^{-1} (○), 0.4 kV mm^{-1} (□) and 0.6 kV mm^{-1} (△).

exponential analysis versus $1/t_{pulse}$ for three different values of excitation voltages. The data show that there is a clear tendency of increasing $<\tau_{rel}>$ with increasing t_{pulse}, whereas no overall systematic dependence on excitation voltage can be deduced within the employed voltages. Linear regression of $<\tau_{rel}>$ versus $1/t_{pulse}$ to infinite pulse duration yields a value of $<\tau_{rel}>_0 = 61\,\mu s$. In principle, this should correspond to the situation where all possible orientational modes that contribute to the birefringence are fully excited.

Figure 5 shows the estimated values of the stretched relaxation exponents β versus $1/t_{pulse}$ using excitation voltage as a parameter. In contrast to $<\tau_{rel}>$, β shows an increasing value for decreasing excitation voltage below $0.4 kV mm^{-1}$. The excitation voltage of $0.2 kV mm^{-1}$ furthermore yields a decrease in β from 0.75 for $t_{pulse} = 0.15$ms to 0.64 for $t_{pulse} = 0.9$ms. Such a decrease in β indicates a broader distribution of relaxation times. Combined with the finding that $<\tau_{rel}>$ increases with increasing pulse length, this indicates that excitation of the slower modes is the most likely reason for this result.

Chain length heterogeneity makes it more difficult to interpret the obtained rotational relaxation times than for homogeneous samples. In order to evaluate the experimental relaxation times, we proceeded as follows: The experimentally determined contour length distribution of schizophyllan M-2 (Stokke *et al.*, 1993) was used as a basis to calculate a spectrum of rotational relaxation times assuming that all species were fully oriented. The spectrum was estimated assuming that the polymer can be modelled as a rigid cylinder employing the Broersma equation (Fig. 6, continuous line). This yielded a monomodal distribution with relaxation times from 2 to 1500μs. Modelling the chains with a given persistence length, L_{pe}, was carried out using the Yoshizaki & Yamakawa (1980) approach. We found that the calculated relaxation time spectra shift towards shorter times for decreasing L_{pe}. For L_{pe}s equal to 150

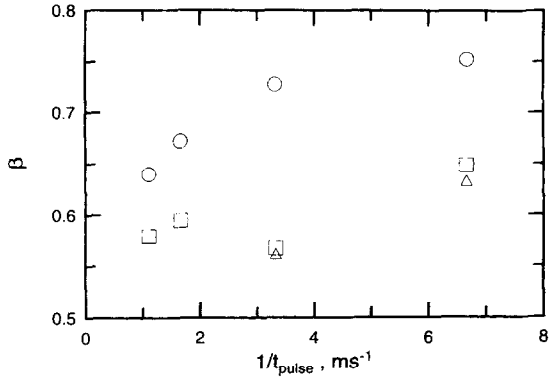

Fig. 5. Stretched exponential parameter β versus the inverse of excitation pulse length for 0.5 mg/ml schizophyllan M-2. The symbols denote excitation field strengths of 0.2 kV mm^{-1} (○), 0.4 kV mm^{-1} (□) and 0.6 kV mm^{-1} (△).

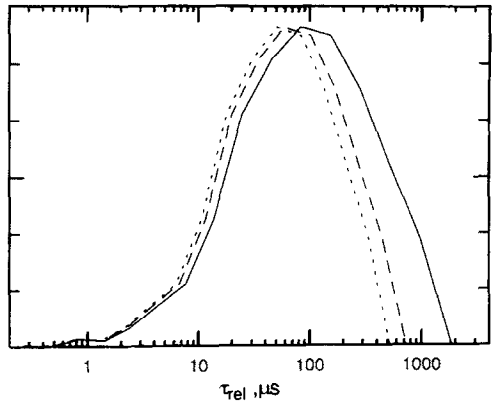

Fig. 6. Relaxation times spectra calculated using the observed contour length distribution and assuming full orientation of all species for the rod-like limit (———) and assuming a persistence length of 150 nm (– – – –) and 100 nm (·····), respectively.

and 100nm (Fig. 6), no modes with relaxation times longer than 700 and 500μs, respectively, were observed. Comparison of these calculated relaxation spectra with those obtained using the CONTIN analysis reveals the following features: Both CONTIN spectra are to a large extent consistent with those calculated using the reported contour length distribution, and the main observed peak shown in Fig. 2(b) coincides with the calculated spectrum. Based on the relaxation spectra derived from the contour length distribution, we know of no reason to expect any bimodal relaxation spectra of the type yielded by the CONTIN-analysis. We therefore find it questionable to pursue such comparisons of the spectra shown in Figs 2 and 6 in order to try to obtain estimates of the persistence lengths of schizophyllan.

Comparison of the calculated relaxation spectra (Fig. 6) and the results of the stretched exponential analysis (eqn (2)) was done by calculating composite birefringence relaxation curves using the estimated relaxation spectra from the contour length distribution and subsequently subjecting these composite curves to the same analysis as the experimental data. The stretched exponential analysis of the composite birefringence curves yielded the same good fits as observed for the experimental data (Fig. 3). This analysis of the composite data yielded β-coefficients ranging from 0.71 (assuming $L_{pe} = 50$nm) to 0.56 (assuming stiff cylinder) which are in the same range as the results obtained from the analysis of the experimental data (Fig. 5). Figure 7 depicts the correlation between $<\tau_{rel}>$ and assumed L_{pe} obtained using the stretched exponential analysis on the composite relaxation curves. Using this correlation, the observed $<\tau_{rel}> = 61\,\mu s$, indicates a persistence length of $L_{pe} = 70$nm for schizophyllan. This value of L_{pe} is on the low side of previously reported values for L_{pe} of schizophyllan, which range from 100 to 200nm. These results were deduced by fitting the worm-like chain model parameters to intrinsic viscosity or sedi-

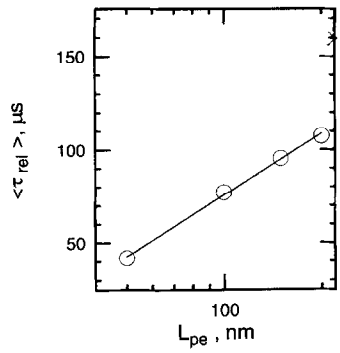

Fig. 7. Average relaxation time $<\tau_{rel}>$, obtained using stretched exponential analysis versus assumed persistence length (○) for the composite decay calculated from the spectra of relaxation times shown in Fig. 6. The rod-like limit is denoted by an arrow (→).

mentation coefficients vs molecular weight data (Norisuye *et al.*, 1980; Yanaki *et al.*, 1980). Measurement of the intrinsic dynamic viscoelastic moduli over an extended frequency range has been reported to yield $L_{pe}=91$nm (Carriere *et al.*, 1985). Generally, the uncertainties resulting from using the whole molecular weight distribution without considering any preferential orientation for certain regions within the size distribution, can result in the observed deviations of L_{pe}. The finding that L_{pe} determined by TEB is smaller than other methods, indicates that the molecular species within the distribution with the highest molecular weight are not fully oriented by the excitation pulse. This appears to be the case despite the fact that extrapolation to long pulse duration (Fig. 4) was used to extract L_{pe} from the relaxation spectra.

The above results were obtained within 4h after centrifugation of a 0.5mg/ml M-2 solution. The rotational relaxation time determined after dissolution, but prior to the centrifugation, yielded 15–25% larger values for $<\tau_{rel}>$ than identical excitation pulses after the centrifugation. Likewise, it was found that $<\tau_{rel}>$ after centrifugation increased slowly to the same level as prior to centrifugation. Storage for 2days yielded the same rotational relaxation time as prior to centrifugation. These results are in agreement with the enhanced solubility reported for schizophyllan in 0.01N NaOH compared to neutral water (Yanaki & Norisuye, 1983). Reduction of the concentration from 0.5 to 0.3mg/ml after centrifugation yielded no significant change in $<\tau_{rel}>$ at identical excitation pulses.

Reduction of the molecular weight from $M_w=437\times10^3$gmol^{-1} to 134×10^3gmol^{-1} by using sample U-1 resulted in a reduction in $<\tau_{rel}>$. The analysis of the decay for the U-1 sample yielded a relaxation time that was comparable to that observed for propylene carbonate. The limitations in the experimental setup therefore prevented any quantitative determination of the molecular weight dependence of $<\tau_{rel}>$ using this rather low M_w sample.

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