TRIPLE HELICES OF β-1,3-D-GLUCANS SCHIZOPHYLLAN AND SCLEROGLUCAN IN AQUEOUS SOLUTION

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by

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Toshio Yanaki

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CONTENTS

CHAPTER I

INTRODUCTION

I-1. General Introduction

Polysaccharides are the most abundant of the constituents of living organisms and a major class of macromolecules essential to life.[1,2] They are either ionic or nonionic. Nonionic (or neutral) polysaccharides are classified into homo- and hetero-groups, depending on whether they consist of identical or different sugars. The former is exemplified by D-glucans, D-mannans, D-fructans, and D-xylans. Among others, D-glucans are best known and most abundant in nature, and can be classified into α-1,2-, β-1,2-, α-1,3-, β-1,3-, α-1,4-, β-1,4-, α-1,6-, and β-1,6-types according to the mode of main chain glucosidic linkage. Starch and cellulose pertaining to our life are the well-known examples of α-1,4- and β-1,4-D-glucans, respectively.

In the past decade, water-soluble, microbial β-1,3-D-glucans have received much attention of polysaccharide chemists and pharmacologists, because of their

industrial use as chemical agents, food additives, drugs, and so on. For example, schizophyllan and lentinan are now under extensive investigation for their promise as anticancer drugs, and Polytran scleroglucan is being used as viscosity-enhancing and suspending agents, cosmetics, food additives, and an enhanced-oil recovery agent.[3] This situation has directed the interest of polysaccharide chemists toward characterization of β-1,3-D-glucans in dilute aqueous solution. However, reported solution studies are yet limited to fragmentary hydrodynamic and spectroscopic measurements. No attempt has been made to determine the absolute molecular weight of β-1,3-D-glucan, except the work of Hirano et al.,[4] who made light scattering and viscosity measurements on a series of curdlan fractions in 1:1 water-diluted cadoxen; curdlan is a water-insoluble β-1,3-D-glucan with no side chain.[5]

The primary purpose of this thesis is to characterize two industrially important β-1,3-D-glucans, schizophyllan and Polytran scleroglucan, in aqueous solution, using standard techniques in polymer solution studies such as light scattering, sedimentation equilibrium and velocity, and viscosity.

I-2. Schizophyllan and Polytran Scleroglucan

Schizophyllan is elaborated extracellularly by the fungus Schizophyllum commune (Suyehiro-take). It was discovered in a culture medium of the fungus by Kikumoto et al.[6] about 15 years ago. Nowadays, its samples are routinely produced by Taito Co. The repeating unit of schizophyllan determined by Kikumoto et al.[7] is shown in Figure I-1.

Komatsu et al.[8] were the first to find that an aqueous solution of schizophyllan has a host-mediated

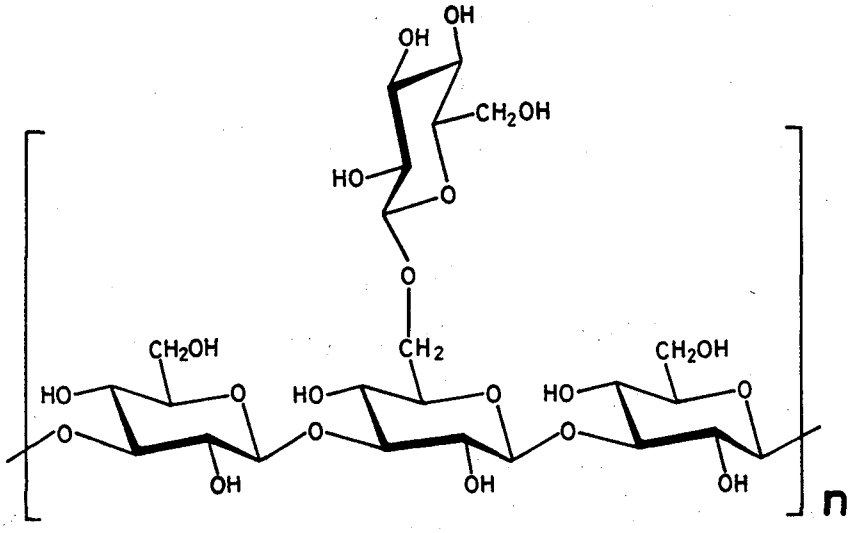

Figure I-1. Repeating unit of schizophyllan.

antitumor activity against tumors of mice (Sarcoma-37, Sarcoma-180, and Ehrlich carcinoma) or Yoshida's tumor of rats. However, they had difficulties in administrating the solution intra-abdominally, intramuscularly, or intravenously, because of an unusually high viscosity of aqueous schizophyllan. Thus, subsequent progress in pharmacological studies on schizophyllan was delayed.

The problem was resolved by Tabata et al.,[9] who found that when sonicated, the viscosity of aqueous schizophyllan solutions could be reduced remarkably, with the chemical structure and antitumor activity of the polymer remaining intact. Although observed viscosity reductions suggested chain scission of native (i.e., unsonicated) schizophyllan, need for molecular weight data on both native and sonicated samples was apparent for direct confirmation of the suggestion. This was the incentive to the present study.

Our molecular weight data, presented in the following chapter, indeed show that native schizophyllan is fragmented to lower-molecular weight chains by sonication. This finding enabled us to investigate the molecular weight dependence of the solution properties

of schizophyllan by standard techniques employed in studying synthetic polymers and thus to deduce its conformation in dilute solution. It was found that Polytran scleroglucan can be studied also by the same techniques.

Scleroglucan is the general name for capsular polysaccharides produced by various species of the genus Sclerotium. Its first example was found by Johnson et al.[10] in 1963, and the species producing the polysaccharide was identified four years later to be Sclerotium glucanicum by Halleck.[11] The scleroglucan from this particular fungus consists of the same repeating units as that of schizophyllan shown in Figure I-1. It is only from this fact that scleroglucans are usually considered the same kind of polysaccharide as schizophyllan. However, this must be accepted with reservation, since the repeating units of scleroglucans from fungi other than Sclerotium glucanicum are not yet known. Polytran scleroglucan, produced by Sclerotium rolfsii, is one of such yet unidentified scleroglucans, and commercially available under the trade name of Polytran (Ceca S. A., France).

I-3. Antitumor Activities of β-1,3-D-Glucans

Besides schizophyllan and lentinan, there are several β-1,3-D-glucans[12-16] having host-mediated antitumor activities against Sarcoma 180, but little is known about the molecular mechanism of their tumor inhibition or even what is likely to be the primary factor for antitumor activities. Table I-1 presents typical bioassay data for β-1,3-D-glucans with or without β-1,6-D-glucose side chains. Here, the tumor inhibition ratio ξ is defined by

Table I-1. Antitumor activities of various β-1,3-D-glucans against Sarcoma 180

Name	ξ	Complete regression	Literature
Schizophyllan	1.0	8/10	8
Polytran scleroglucan	0.97	7/10	a
Curdlan	0.99	5/6	12
Pachyman	0	0/8	17
Lentinan	1.0	10/10	18
U-pachyman	0.91	5/10	13
Laminalan	0.02	0/10	15

[a] T. Yanaki, unpublished data.

$$\xi = \frac{w_c - w_t}{w_c} \qquad (1\text{-}1)$$

with w_t and w_c the average tumor weights of the glucan-treated and untreated groups of mice, respectively; and the complete regression is defined as the number of tumor free, glucan-treated mice* relative to the total number of the glucan-treated mice. As can be seen from the table, pachyman[17] and laminaran[15,16] have virtually no antitumor activity, suggesting that the backbone chemical structure cannot be the primary factor for antitumor activities of β-1,3-D-glucans.

Sasaki et al.[12,19] found curdlan and lentinan to lose their antitumor power when the molecular weight (estimated by end group analysis) was lowered below $5 - 8 \times 10^3$. These authors[19] and also Saito et al.,[20] investigating the conformation of lentinan fractions in dilute aqueous sodium hydroxide by visible absorption and ^{13}C-NMR, concluded that the loss of antitumor potency of lentinan is caused by an order-disorder conformation change of the glucan accompanying the decrease in molecular weight. This conclusion along

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* Mouse having a tumor weight less than 0.05 g.

with some other pieces of information[15,17,21,22] led Chihara[16] to hypothesize that a certain ordered structure of a glucan in aqueous solution is primarily responsible for its antitumor activity, but the 'ordered' structure has remained not specified.

I-4. Scope of This Work

Preliminary solubility tests showed that schizophyllan and Polytran scleroglucan are soluble in dimethyl sulfoxide (DMSO). To start with, we measured viscosities of schizophyllan in this solvent and pure water, and found that intrinsic viscosities [η] of both native and sonicated samples in water were appreciably higher than those of the same sample in DMSO. At that time, we thought that this difference could be due to difference in molecular conformation in the two solvents, i.e., an extended rodlike conformation in water and a spherical random coil in DMSO. However, we came to know that things were not quite as simple as that when molecular weight data in the two solvents became available. The weight-averege molecular weight of each sample in water was approximately three times that in DMSO. This indicated that schizophyllan in water

consists of three chains.

Our final conclusion was that this glucan dissolves as a rodlike triple helix in water and as a single random coil in DMSO. The hydrodynamic characterization of schizophyllan, which led to this conclusion, is described in the following chapter. Chapter III is concerned with the melting or dissociation of the schizophyllan triple helix into three single chains in water-DMSO mixtures at 25°C and in pure water at elevated temperature. In Chapter IV, the antitumor activity of aqueous schizophyllan is investigated in relation to Chihara's hypothesis mentioned above.

Chapters V and VI are devoted to a study on Polytran scleroglucan solutions. The former deals with the question whether this glucan is identical to schizophyllan in regard to chemical structure and dilute-solution behavior. The latter concerns concentrated aqueous solutions. Actually, the study described in Chapter VI was motivated by very recent work of Van et al.,[23] who found from microscopic observations and polarimetric measurements that schizophyllan in aqueous solution forms a cholesteric liquid crystal when the concentration is higher than

a certain critical value. The last chapter VII summarizes the main results and conclusions from the present work.

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Antitumor Activity of Degraded Products of Lentinan, Its Correlation with Molecular Weight, Gann, 67, 191 - 195 (1976).

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CHAPTER II

TRIPLE HELIX OF SCHIZOPHYLLAN IN DILUTE AQUEOUS SOLUTION

II-1. Introduction

Schizophyllan, a nonionic, extracellular β-1,3-D-glucan produced by the fungus Schizophyllum commune,[1] is soluble in water and DMSO. This chapter presents molecular weight, viscosity, and sedimentation velocity data from which it can be concluded that this polysaccharide disperses as rodlike trimers having a triple-stranded helical structure in water and as single randomly coiled chains in DMSO. The pitch, diameter, and stiffness of the triple helix are estimated, using the hydrodynamic theories of Yamakawa et al.[2-5]

II-2. Experimental

II-2-1. Samples

A native schizophyllan sample N-1 and 10 sonicated samples S-65, S-45, S-1, B, S-148, S-144, H, S-166, E, and S-164 were prepared in the following way.[6]

An aqueous solution (0.2 - 0.3 % polymer) of a crude schizophyllan sample (Taito Co.) was filtered through diatomite and treated with activated charcoal. To this filtrate, excess acetone was added to precipitate the polymer. The sample so purified was dissolved in water at a concentration of 0.1 - 1.0 %, and the solution was exposed to 19.5 kHz sound (Kaijo Denki, Model TA-6280N). The duration of ultrasonic irradiation was varied from 5 to 120 h on the basis of the preliminary sonication data shown in Figure II-1. Each sonicated

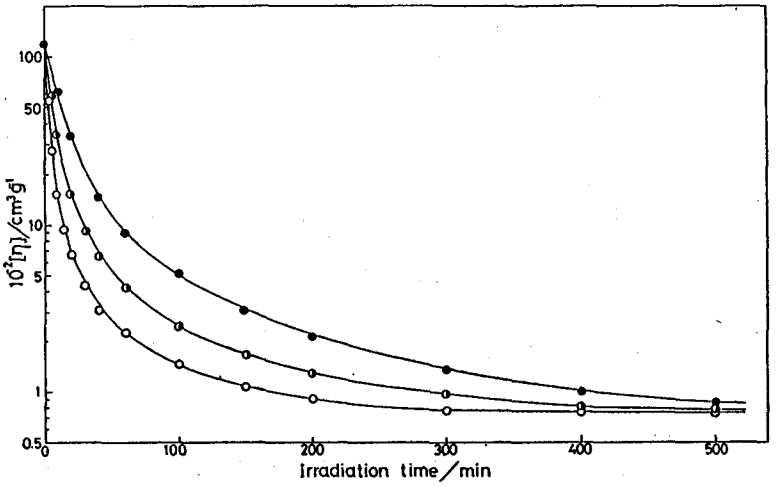

Figure II-1. Changes in [η] of schizophyllan that occurred when aqueous solutions of concentrations 0.10 (○), 0.20 (◑), and 0.40 (●) x 10^{-2}g cm^{-3} were sonicated at 19.5 kHz.

solution was passed through a Millipore filter (type HA) after being deionized with ion exchange resins (Nippon Orugano, IRA 402 and IR 120B). Sample N-1 was prepared in the same way but without sonication. It has been reported[6] that sonication gave rise to no detectable changes in chemical structure or in ^{13}C-NMR and infrared spectra of native schizophyllan.

The native sample N-1 was used as it stood. Each of the sonicated samples S-65, S-45, S-1, B, S-148, S-144, H, S-166, E, and S-164 was separated into three to six parts by fractional precipitation with water as the solvent and either acetone or ethanol as the precipitant. The lowest-molecular weight fraction from each sample in pure water exhibited polyelectrolytic viscosity behavior, and hence was discarded. Middle fractions designated below as S-65-2, S-45-4, S-1-2, B-4, S-148-2, S-144-2, H-3, S-166-4, E-4, and S-164-3 were chosen for the present study. These 10 fractions were freeze-dried from aqueous solutions, while sample N-1 was dried in vacuo and crushed into powder. Before use, each sample was further dried overnight in vacuo at room temperature.

II-2-2. Molecular Weight Determination

a. Weight-Average Molecular Weight

Sedimentation equilibria of schizophyllan samples in water and DMSO at 25°C were studied in a Beckman Spinco Model E ultracentrifuge equipped with an electronic speed-control system. A filled Epon 30 mm double-sector cell was used for samples N-1, S-65-2, S-45-4, S-1-2, B-4, S-148-2, and S-144-2 in water, and a Kel-F 12 mm double-sector cell for samples H-3, S-166-4, E-4, and S-164-3 in water and for samples S-148-2, S-144-2, S-166-4, and S-164-3 in DMSO. The liquid column was adjusted to 1.2 - 2 mm, but for the highest-molecular weight sample N-1, it was reduced to 0.9 mm at the expense of the accuracy of measurement to accelerate the attainment of sedimentation equilibrium; even with this length it took about four days for the equilibrium to be attained. The rotor speed was set at 1200 - 14000 rpm, depending on test sample and solvent. Rayleigh fringe patterns were photographed on Kodak spectroscopic plates and read on a Nikon Shadowgraph Model 6 to the accuracy of ± 0.5 %.

Sedimentation equilibrium data were analyzed by the equation[7]

$$M_{app}^{-1} = M_w^{-1} + 2A_2(1 + \delta_1)\bar{c} + \cdots \qquad (2\text{-}1)$$

where

$$M_{app} = (c_b - c_a)/\lambda c_0 \qquad (2\text{-}2)$$

$$\bar{c} = (c_a + c_b)/2 \qquad (2\text{-}3)$$

$$\delta_1 = (\lambda^2 M_w M_z/12)(M_z/M_w - 1) + O(\lambda^4) \qquad (2\text{-}4)$$

with

$$\lambda = (1 - \bar{v}\rho_0)(r_b^2 - r_a^2)\omega^2/2RT \qquad (2\text{-}5)$$

Here, M_w and M_z are the weight-average and z-average molecular weights of the polymer, respectively, A_2 the second virial coefficient of the solution, c_b and c_a the equilibrium polymer mass concentrations at the bottom and meniscus of the liquid column, respectively, c_0 the initial polymer mass concentration, $\bar{v}$ the partial specific volume of the polymer, ρ_0 the density of the solvent, r_b and r_a the radial distances from the center of rotation to the bottom of the cell and the meniscus, respectively, ω the angular velocity of the rotor, R the gas constant, and T the absolute temperature. In evaluating A_2, the correction term δ_1 was estimated with only the first term in eq 2-4 retained, but it was

neglected for sample N-1 in water.

The weight-average molecular weights of samples N-1 and S-1-2 in DMSO at 25°C were determined by light scattering, using a Fica 50 automatic light-scattering photometer (see Chapter V for the experimental details).

b. z-Average Molecular Weight

Values of M_z of samples S-65-2, S-45-4, S-1-2, S-148-2, S-144-2, H-3, S-166-4, E-4, and S-164-3 in water and samples S-148-2, S-144-2, S-166-4, and S-164-3 in DMSO at 25°C were estimated from sedimentation equiliblium data. The data analysis was made by an equation derived in this work on the same assumption[7] as used in deriving eq 2-1. It reads

$$Q = (M_w/M_z)[1 + 2A_2M_w(1 + \delta_2)\bar{c} + \cdots] \qquad (2\text{-}6)$$

where

$$Q = \frac{(c_b - c_a)^2}{c_0(r_b^2 - r_a^2)[(\partial c/\partial r^2)_{r=r_b} - (\partial c/\partial r^2)_{r=r_a}]} \qquad (2\text{-}7)$$

$$\delta_2 = (\lambda^2 M_w M_z/12)(3M_{z+1}/M_w - 2M_z/M_w - 1) + O(\lambda^4) \qquad (2\text{-}8)$$

and M_{z+1} is the $(z + 1)$-average molecular weight.

Experimental values of Q were plotted against $\bar{c}$ and extrapolated graphically to infinite dilution. The primary factor governing Q was the values of $\partial c/\partial r^2$ at the meniscus and the bottom. Since these were estimated graphically, the M_z values determined were correct only to ± 10 %.

c. Partial Specific Volume

Densities ρ of schizophyllan in water and DMSO at 25°C were determined by a Lipkin-Davison pycnometer of

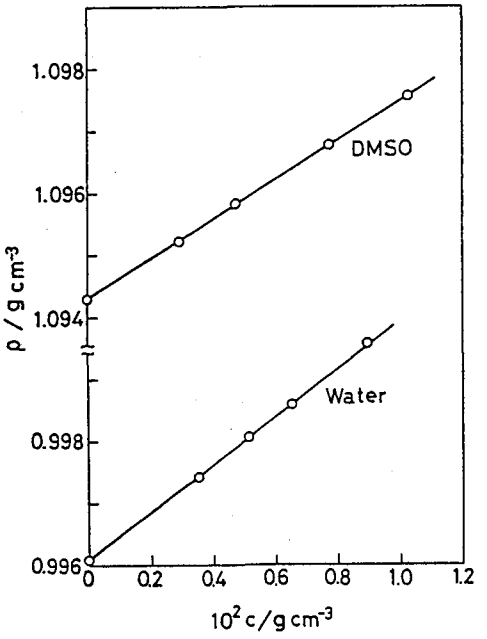

Figure II-2. Densities ρ of aqueous or DMSO solutions of schizophyllan as a function of c.

30 ml capacity. Figure II-2 shows that the measured ρ in water and DMSO vary linearly with c in the concentration range studied. The slopes of the straight lines yield 0.619 and 0.623 $cm^3 g^{-1}$ for the partial specific volumes of schizophyllan in water and DMSO at 25°C, respectively.

d. Specific Refractive Index Increment

Refractive indices of schizophyllan in water and DMSO at 25°C were measured on a modified Schulz-Cantow

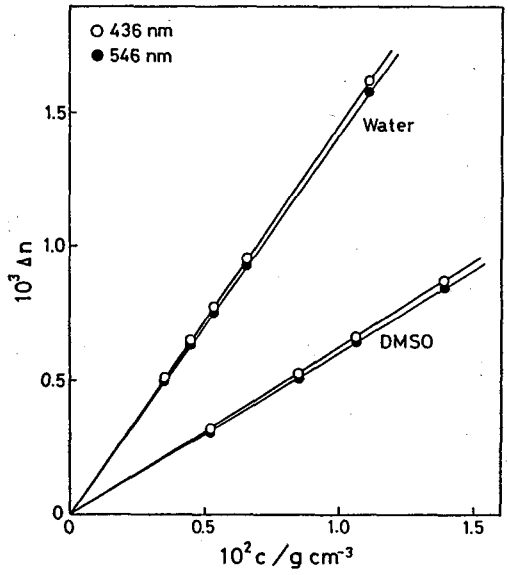

Figure II-3. Excess refractive indices Δn plotted against c for schizophyllan in water and DMSO at 25°C.

differential refractometer. The apparatus was calibrated with aqueous potassium chloride and Kruis' data[8] as the reference. The measured excess refractive indices Δn in the two solvents are plotted against c in Figure II-3. From the indicated straight lines, the specific refractive index increments $(\partial n/\partial c)$ in water at 25°C are determined to be 0.145 cm^3g^{-1} at 436 nm wavelength and 0.142 cm^3g^{-1} at 546 nm, and those in DMSO at 25°C to be 0.0624 cm^3g^{-1} at 436 nm and 0.0611 cm^3g^{-1} at 546 nm.

II-2-3. Sedimentation Velocity

Sedimentation velocities of 11 schizophyllan samples in water at 25°C were measured using a Kel-F 30 mm single-sector cell. The rotor speed was 40000 rpm for sample N-1 and 48000 rpm for the rest. For any solutions the observed schlieren patterns were single-peaked, so that sedimentation coefficients s were evaluated according to the equation[9]

$$\ln r_p = s\omega^2 t + \text{constant} \qquad (2\text{-}9)$$

where r_p is the radial distance from the center of

rotation to the peak position in the schlieren pattern and t, the time of centrifugation. The plots of ln r_p vs. t obtained were strictly linear over the entire range of t studied, and thus s could be determined accurately.

The values of s obtained for a series of c_0 were extrapolated to infinite dilution by use of an empirical relation[9]

$$s^{-1} = s_0^{-1}(1 + k_s c_0) \qquad (2\text{-}10)$$

to evaluate the limiting sedimentation coefficient s_0 and the constant k_s.

II-2-4. Viscometry

Viscosities of four highest-molecular weight samples, N-1, S-65-2, S-45-4, and S-1-2, in water at 25°C were measured using a rotational viscometer[10] of the Zimm-Crothers type and four-bulb capillary viscometers[11] of the Ubbelohde type, since appreciable shear rate effects were anticipated on the intrinsic viscosities $[\eta]$ of these samples.

Dependence of $[\eta]$ on 'apparent' shear rate (estimated

for pure water) is illustrated in Figure II-4, in which the data determined by conventional capillary viscometers of the Ubbelohde type are also shown for comparison. The curves indicate that the zero shear [η] for samples N-1, S-65-2, and S-45-4 can be determined directly by the

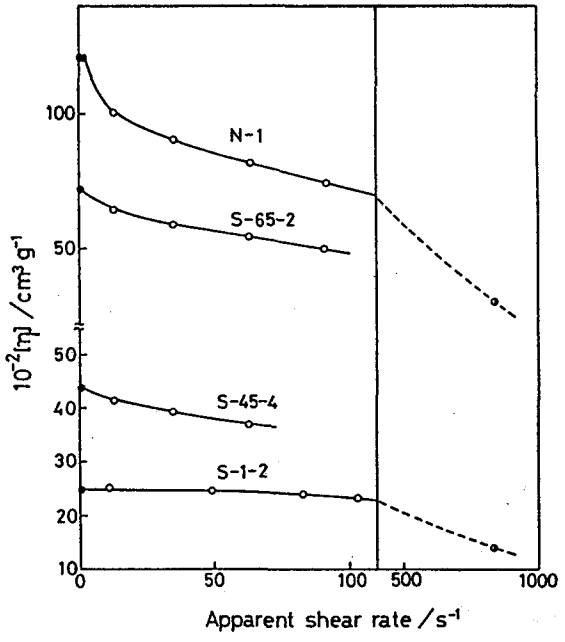

Figure II-4. Dependence of [η] on apparent shear rate for indicated schizophyllan samples in water at 25°C. (●), Zimm-Crother rotational viscometer; (○), low-shear four-bulb capillary viscometers; (◑), conventional Ubbelohde capillary viscometers.

rotational viscometer, and that for sample S-1-2 by extrapolating the four-bulb viscometer data.

On the basis of these data, the zero shear $[\eta]$ of lower-molecular weight samples B-4, S-148-2, S-144-2, H-3, and S-166-4 in water were determined by the four-bulb viscometers. For the two lowest-molecular weight samples E-4 and S-164-3 in water and all samples in DMSO, conventional capillary viscometers of the Ubbelohde type were used.

II-3. Results

II-3-1. Molecular Weights

Figures II-5 and II-6 show plots of M_{app}^{-1} vs. $\bar{c}$ and Q vs. $\bar{c}$ for schizophyllan samples in water and DMSO, respectively. The values of M_w, A_2, and M_z estimated from the straight lines in these figures are summarized in Table II-1. Figure II-7 illustrates light-scattering data for sample N-1 in DMSO at 25°C. The light-scattering values of M_w, A_2, and $\langle S^2 \rangle$ (the mean-square radius of gyration) are also given in Table II-1, along with the values of the molecular weight ratios

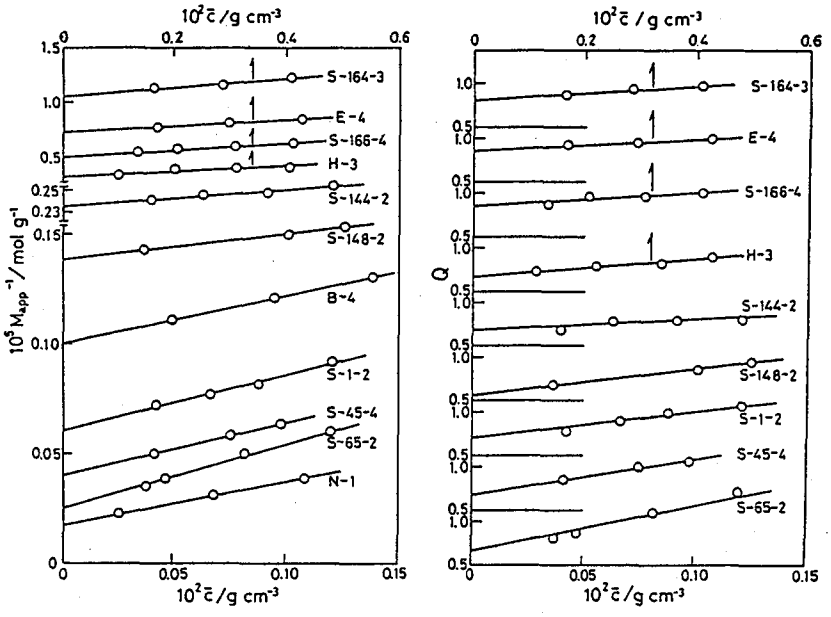

Figure II-5. Plots of M_{app}^{-1} vs. $\bar{c}$ and Q vs. $\bar{c}$ for indicated schizophyllan samples in water at 25°C.

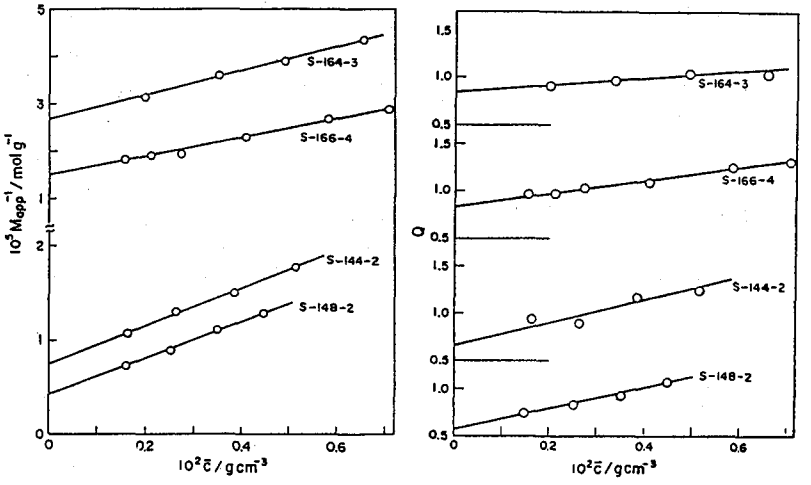

Figure II-6. Plots of M_{app}^{-1} vs. $\bar{c}$ and Q vs. $\bar{c}$ for indicated schizophyllan samples in DMSO at 25°C.

Table II-1. Results from sedimentation equilibrium and light scattering measurements on schizophyllan samples in water and DMSO at 25°C

	In water				In DMSO					M_w(in water)	M_z(in water)
Sample	$10^{-4}M_w$	10^4A_2[a]	$10^{-4}M_z$	M_z/M_w	$10^{-4}M_w$	10^4A_2[a]	$10^{-4}M_z$	M_z/M_w	$\langle S^2\rangle/nm^2$	M_w(in DMSO)	M_z(in DMSO)
N-1	570	1.0	—	—	164[b]	4.05[b]	—	—	7700	3.48	—
S-65-2	400	1.4	610	1.5	—	—	—	—	—	—	—
S-45-4	248	1.2	370	1.5	—	—	—	—	—	—	—
S-1-2	161	1.1	230	1.4	58.5[b]	5.57[b]	—	—	1400	2.75	—
B-4	100	1.1	—	—	—	—	—	—	—	—	—
S-148-2	72.4	0.6	130	1.8	23.3	8.4	41	1.8	—	3.11	3.2
S-144-2	42.9	1.0	62	1.4	13.5	9.8	20	1.5	—	3.18	3.1
H-3	32.0	1.2	48.3	1.5	—	—	—	—	—	—	—
S-166-4	19.6	1.4	23	1.2	6.67	9.6	8.0	1.2	—	2.94	2.9
E-4	13.9	1.6	16.4	1.2	—	—	—	—	—	—	—
S-164-3	9.60	2.2	12	1.3	3.72	12.2	4.4	1.2	—	2.58	2.7

[a] In units of $cm^3 mol\ g^{-2}$.

[b] From light scattering.

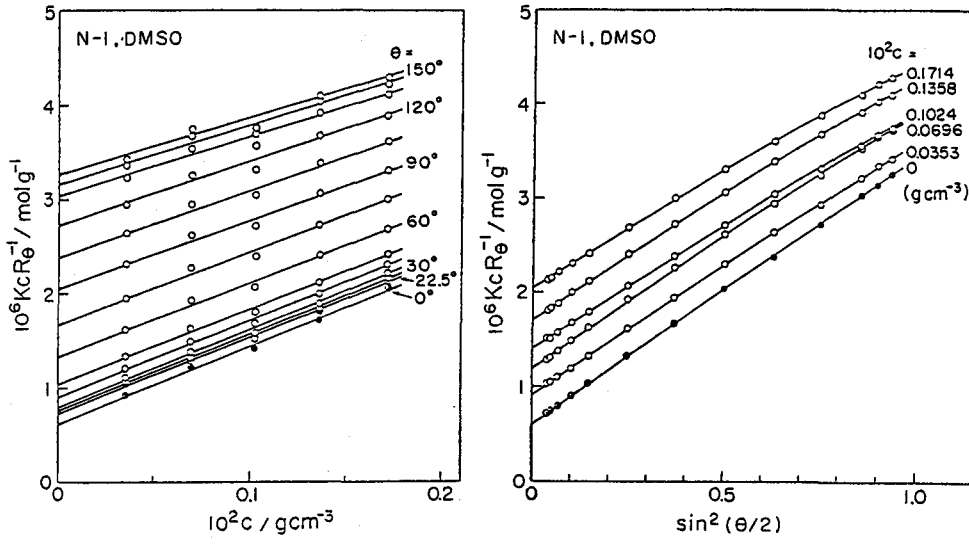

Figure II-7. Light-scattering envelopes for sample N-1 in DMSO at 25°C. K, the optical constant; R_θ, the reduced scattering intensity; θ, the scattering angle.

M_w(in water)/M_w(in DMSO) and M_z(in water)/M_z(in DMSO).

The ratios M_w(in water)/M_w(in DMSO) and M_z(in water)/M_z(in DMSO) in Table II-1 are close to three for all samples examined. The implication of this finding is that the predominant species present in an aqueous solution of schizophyllan is a trimer, because the DMSO data for A_2 and $\langle S^2 \rangle$ in the same table convince us that this polysaccharide is dispersed molecularly in DMSO. It can be shown theoretically that the weight-average molecular weight $M_w(3)$ and the z-average molecular

weight $M_z(3)$ of the solute in a polydisperse solution of trimers are equal to $3M_w(1)$ and $3M_z(1)$, respectively, only when each trimer molecule consists of chains of equal length. Here, $M_w(1)$ and $M_z(1)$ are the corresponding average molecular weights of dissociated monomers. Since this theoretical prediction holds, regardless of the heterogeneity in size of the trimers in solution, we may further conclude that the schizophyllan trimer in water consists of chains of approximately equal molecular weights.

II-3-2. Viscosity-Molecular Weight Relations

The measured values of $[\eta]$ and k' (Huggins' constant) for 11 schizophyllan samples in water and DMSO at 25°C are summarized in Table II-2.

Figure II-8 depicts double-logarithmic plots of $[\eta]$ vs. M_w in these two solvents. The two curves have distinctly different slopes and intersect at an M_w of about 1.5×10^5, suggesting that the shape of the schizophyllan trimer in water is different from that of the single schizophyllan molecule in DMSO. The slope of the curve for water is about 1.7 in the range of M_w below 5×10^5, and gradually decreases with increasing

Table II-2. Results from viscosity and sedimentation velocity measurements on schizophyllan samples in water and DMSO at 25°C

	In water				In DMSO	
Sample	$10^{-2}[\eta]/cm^3g^{-1}$	k'	$10^{13}s_0/s$	$10^{-2}k_s/cm^3g^{-1}$	$10^{-2}[\eta]/cm^3g^{-1}$	k'
N-1	120	0.44	15.0	26.1	4.65	0.35
S-65-2	71.9	0.46	13.0	16.4	—	—
S-45-4	43.7	0.42	11.2	7.66	—	—
S-1-2	25.0	0.43	10.3	4.26	2.19	0.34
B-4	15.4	0.40	9.12	2.55	—	—
S-148-2	9.62	0.42	8.74	2.12	1.14	0.33
S-144-2	4.78	0.44	8.01	1.29	0.813	0.31
H-3	3.01	0.42	7.36	1.17	—	—
S-166-4	1.32	0.42	6.57	0.72	0.546	0.41
E-4	0.736	0.43	6.09	0.52	—	—
S-164-3	0.401	0.43	5.25	0.34	0.317	0.41

M_w in the higher-molecular weight range. This behavior implies that the schizophyllan trimer can be modeled as an almost completely rigid rod below and as a semiflexible rod above $M_w \sim 5 \times 10^5$.

On the other hand, the curve for DMSO solutions in Figure II-8 is linear with a slope of 0.68. This slope value indicates that the schizophyllan monomer in DMSO is a random coil swollen by excluded-volume effect.

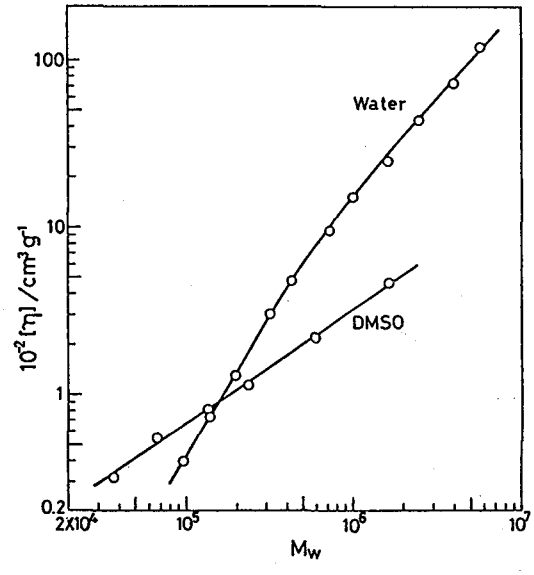

Figure II-8. Double logarithmic plots of $[\eta]$ vs. M_w for schizophyllan in water and DMSO at 25°C.

II-3-3. Sedimentation Coefficient

Figure II-9 shows the concentration dependence of s^{-1} for schizophyllan samples in water. The plot for the highest-molecular weight sample N-1 is almost linear below $c_0 = 2 \times 10^{-4}$ g cm^{-3}, allowing the intercept to be determined fairly accurately. The values of s_0

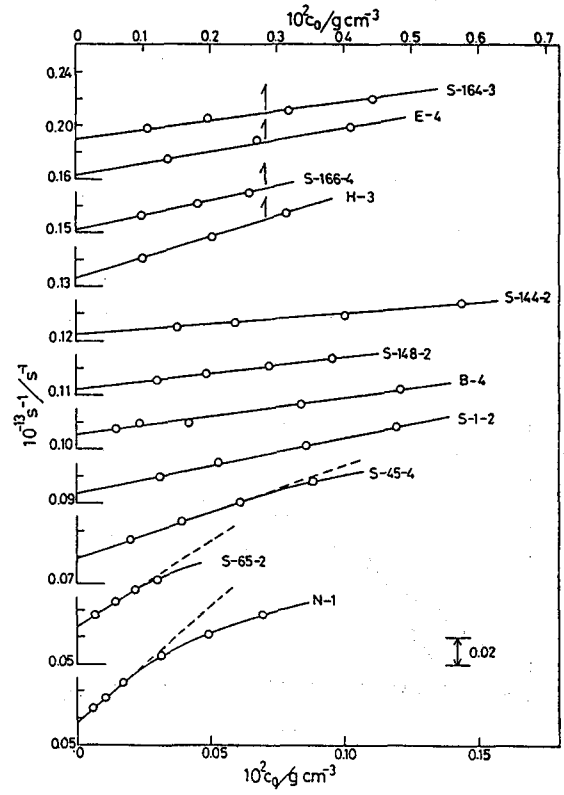

Figure II-9. Concentration dependence of s^{-1} for schizophyllan samples in water at 25°C.

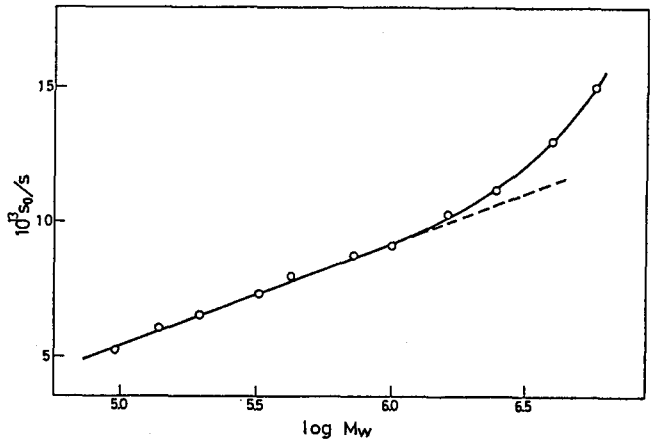

Figure II-10. Molecular weight dependence of s_0 for schizophyllan in water at 25°C.

and k_s evaluated from the intercepts and slopes of the indicated straight lines are presented in the fourth and fifth columns of Table II-2. These s_0 values, plotted against log M_w in Figure II-10, follow a straight line for $M_w \leq 10^6$. This linear behavior is another evidence for the rigid rod nature of the schizophyllan trimer in water, since it conforms to the theoretical prediction[3] for long straight rods (see eq 2-12). The upswing of the curve for M_w higher than 10^6 is consistent with the finding from $[\eta]$ that the rodlike trimer exhibits flexibility at these high molecular weights.

II-4. Discussion

II-4-1. Molecular Model Building

The hydrodynamic data described above suggest that the molecular chains in the schizophyllan trimer are arranged in a regular three-dimensional structure. Bluhm and Sarko[12] and Deslandes et al.[13] found from x-ray studies on lentinan and curdlan, respectively, that these β-1,3-D-glucans in the crystalline region have essentially the same triple helical structure as that proposed by Atkins et al.[14] for a β-1,3-D-xylan from x-ray data. Each chain in the triple helix of Atkins et al. comprises six xylose rings in a pitch of 1.836 nm (0.306 nm per ring). We suspected that the schizophyllan trimer may also have a regular structure similar to the triple helix of Atkins et al. However, we asked ourselves whether the bulky side chains, β-1,6-D-glucose rings, may prevent three schizophyllan molecules from forming such a structure. To answer this question, we attempted to make a model helix according to Atkins et al.

Figure II-11 illustrates our molecular model for the schizophyllan trimer. Each chain in the trimer

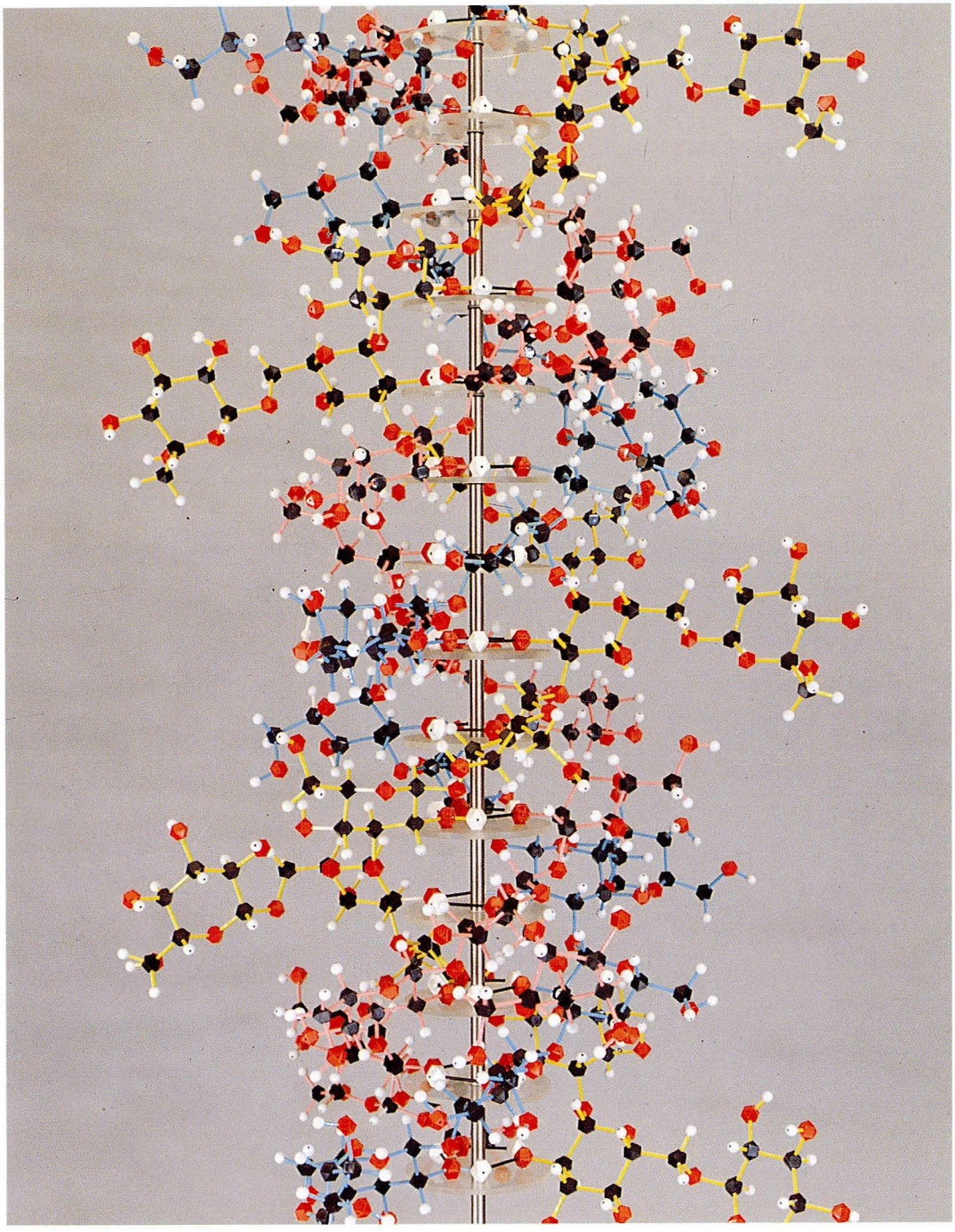

Figure II-11. Molecular model of the schizophyllan triple helix.

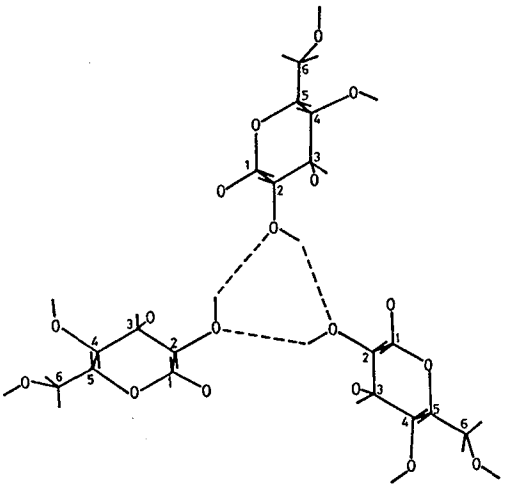

Figure II-12. Cyclic triad of hydrogen bonds among three schizophyllan chains. Hydrogen bonds are represented by dashed lines.

winds a right-handed helix and has exactly six residues per turn. The three helices are stabilized by interchain hydrogen bonds between the OH-groups attached to C-2 carbons, as can be seen from Figure II-12; the hydrogen bonds form cyclic triads at the center of the structure. The model clearly shows that the CH_2OH groups and the side chains do not impede three schizophyllan chains to form the triple helix of Atkins et al., but simply add to the diameter d of the helix. The diameter and pitch (per residue) of the model triple helix of schizophyllan are 2.5 - 3.0 nm and 0.30 nm, respectively.

II-4-2. Triple Helix of Schizophyllan

Yamakawa's expression[2] for [η] of a long straight cylinder may be written

$$M^2/[\eta] = \frac{45M_L^3}{2\pi N_A}\,[\ln M - 0.6970 - \ln(d\,M_L)] \qquad (2\text{-}11)$$

where M is the molecular weight of the polymer, M_L the molar mass per unit cylinder length, and N_A Avogadro's constant. The values of $M_w^2/[\eta]$ for five lowest-molecular weight samples in aqueous solution are plotted against log M_w in Figure II-13 according to eq 2-11.

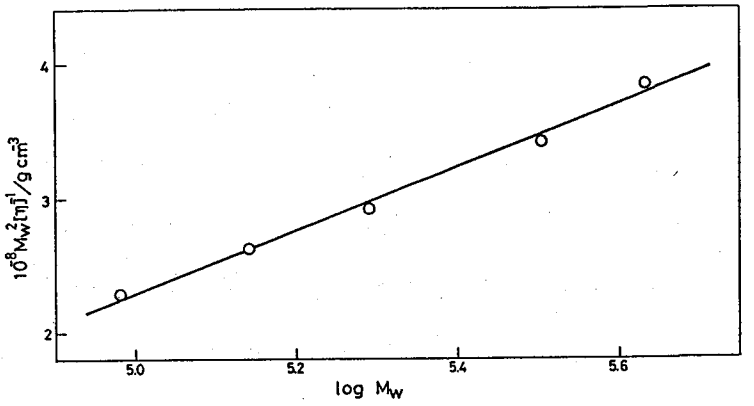

Figure II-13. Plot of $M_w^2/[\eta]$ vs. log M_w for schizophyllan in water at 25°C.

The plotted points can be fitted by a straight line, with a maximum deviation less than 2.5 %. If analyzed in terms of eq 2-11, the slope and intercept (at log M_w = 0) of the straight line yield 2100 ± 100 nm^{-1} for M_L and 2.6 ± 0.6 nm for d. The large uncertainty in d is due to the fact that the theoretical expression for [η] is not sensitive to d.

Yamakawa-Fujii's expression[3] for s_0 of a long straight cylinder is

$$s_0 = \frac{(1 - \bar{v}\rho_0)M_L}{3\pi\eta_0 N_A} [\ln M + 0.3863 - \ln(d\, M_L)] \qquad (2\text{-}12)$$

where η_0 is the solvent viscosity. This equation combined with the linear relation between s_0 and log M_w in Figure II-10 gives M_L = 2200 ± 100 nm^{-1} and d = 2.6 ± 0.4 nm. These M_L and d values are in substantial agreement with those estimated above from the [η] data. Further, the d value of 2.6 nm is quite close to that of the model triple helix of schizophyllan.

Each repeating unit of the main chain of schizophyllan contains three β-1,3-D-glucose residues (see Figure I-1 in Chapter I). Thus, the contour

length h per main chain residue of the schizophyllan trimer is related to M_L by

$$h = (M_0/3) \; / \; (M_L/3) \qquad (2\text{-}13)$$

with M_0 the molar mass of the schizophyllan repeating unit (= 648 g mol^{-1}). Substitution of 2150 ± 150 nm^{-1} for M_L (which is the average of the estimates from s_0 and $[\eta]$) into eq 2-13 yields 0.30 ± 0.02 nm for h, which agrees with the pitch per residue (0.30 nm) of the model triple helix of schizophyllan. Thus, it may be concluded that the three schizophyllan chains in a rodlike trimer form a triple helical structure.

Our h value may be compared with 0.306 nm for the right-handed triple helix of a β-1,3-D-xylan,[14] 0.29 nm (right-handed) or 0.33 nm (left-handed) for the triple helix of lentinan[12] (a β-1,3-D-glucan), and 0.294 nm for curdlan,[13] all in the crystalline region. The agreement allows us to conclude further that the triple helical structure of schizophyllan in dilute solution is very much similar to those of these other polysaccharides in the crystalline state.

II-4-3. Flexibility of the Triple Helix

The viscosity and sedimentation velocity data presented above suggest that the schizophyllan triple helix with $M_w > 10^6$ can be better represented by a wormlike cylinder than by a straight cylinder. The hydrodynamic theories of Yamakawa and co-workers[3-5] for wormlike cylinders express both s_0 and $[\eta]$ as functions of M_L, d, and persistence length q. With M_L and d fixed to 2150 nm^{-1} and 2.6 nm, respectively, we sought a q which allows the Yamakawa-Fujii theory[3] for s_0 and the Yamakawa-Yoshizaki theory[5] (a modification of the Yamakawa-Fujii theory[4]) for $[\eta]$ to fit our experimental data.

The curves in Figure II-14 show theoretical values of $[\eta]$ (on a logarithmic scale) and s_0 (on a linear scale) calculated for q = 100, 200, 300 nm and ∞. It can be seen that the q value of 200 nm leads to the closest agreement between theory and experiment. Similar analyses allowing M_L and d to vary within the range of uncertainty (± 150 nm^{-1} for M_L and ± 0.4 nm for d) yielded q for the schizophyllan triple helix in a range from 170 to 230 nm. The q value in this range is comparable to or even slightly larger than 160 - 180 nm

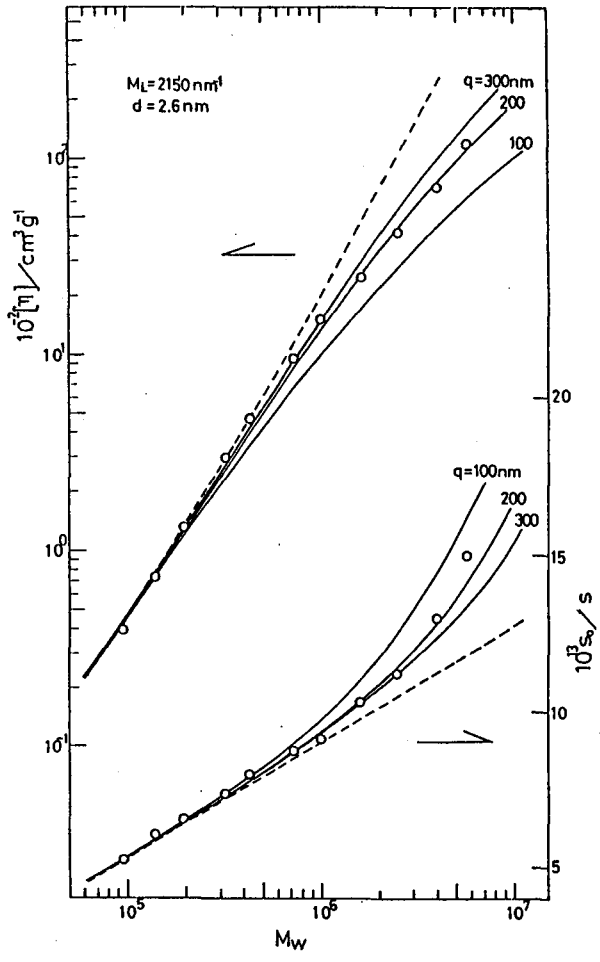

Figure II-14. Comparison of the experimental s_0 and $[\eta]$ for schizophyllan with the theoretical values for wormlike cylinders[3,5] calculated for q = 100, 200, 300 nm, and ∞ (the dashed lines) with M_L and d fixed to 2150 nm^{-1} and 2.6 nm, respectively.

for native collagen,[15] a triple-helical biopolymer, and much larger than 60 nm for double-stranded DNA,[16] indicating a very high stiffness of the schizophyllan triple helix.

Figure II-15 shows the molecular weight dependence of the Flory-Scheraga-Mandelkern parameter[17,18] β defined by

$$\beta = \frac{N_A \eta_0 s_0 [\eta]^{1/3}}{(1 - \bar{v}\rho_0) M^{2/3} 100^{1/3}} \qquad (2\text{-}14)$$

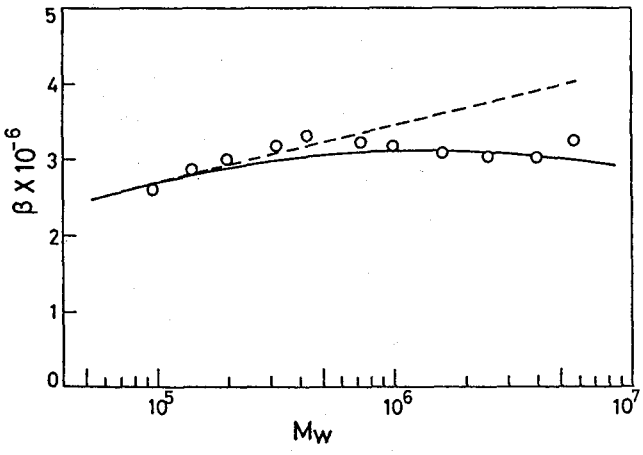

Figure II-15. Flory-Scheraga-Mandelkern parameter β (in conventional units) for schizophyllan in water at 25°C. The solid line refers to the wormlike cylinder with q = 200 nm, M_L = 2150 nm^{-1}, and d = 2.6 nm, and the dashed line to the rigid cylinder with the same M_L and d values.

The solid curve refers to a wormlike cylinder with q = 200 nm, M_L = 2150 nm^{-1}, and d = 2.6 nm, and the dashed line to a rigid cylinder with the same M_L and d values. The former and the experimental values first increase and then gradually decrease with increasing M_w, whereas the dashed curve shows a monotonic increase. The rise in experimental β at lower molecular weights is due to an increasing axial ratio of the rigid triple helix, while the decline at higher molecular weights is due to an increasing bending of the helix.

II-4-4. Conclusions

The major conclusions derived from the study in this chapter are as follows. (i) Schizophyllan dissolves in water as a trimer; (ii) the trimer is a triple helix which has a pitch per residue of 0.30 ± 0.02 nm and a diameter of 2.6 ± 0.4 nm; and (iii) the helix has a high rigidity specified by a persistence length of 200 ± 30 nm. These conclusions were recently confirmed by Kashiwagi et al.,[19] who investigated schizophyllan by light scattering and viscometry in water containing 0.01 N sodium hydroxide.

More recently, Takahashi et al.[20] found from an

x-ray diffraction study that the conformation of schizophyllan in the crystalline state is a 6_1 triple helix with a pitch per residue of 0.3025 nm, which is very close to the value from our solution study. Thus, we see that when dissolved in water, schizophyllan maintains its conformation in the crystalline state.

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CHAPTER III

DISSOCIATION OF THE SCHIZOPHYLLAN TRIPLE HELIX

III-1. Introduction

In the preceding chapter, we showed that schizophyllan dissolves as rodlike trimers having a triple helical structure in water at 25°C and as single, randomly coiled chains in DMSO at the same temperature. In this chapter, we investigate by viscosity, optical rotatory dispersion (ORD), and viscoelasticity what occurs to schizophyllan when its aqueous solution is successively diluted with DMSO at 25°C or heated.

III-2. Experimental

III-2-1. Samples

Three of the sonicated samples studied in Chapter II, S-144-2, S-166-3, and S-164-3, and newly prepared two sonicated samples M-2 and U-1 (see Chapter IV for the preparation of U-1) were used. Their molecular characteristics are summarized in Table III-1.

Table III-1. Molecular characteristics of the schizophyllan samples used

Sample	In water		In DMSO	
	$10^{-2}[\eta]/cm^3g^{-1}$	$10^{-4}M_w$	$10^{-2}[\eta]/cm^3g^{-1}$	$10^{-4}M_w$
M-2	4.79	43.7	0.820	13.6
S-144-2	4.78	42.9	0.813	13.5
S-166-3	1.66	24[a]	0.640	9.1[a]
U-1[b]	0.584	13.4	0.397	4.7
S-164-3	0.401	9.60	0.317	3.72

[a] Estimated from $[\eta]$ using the $[\eta]$ vs. M_w relations for schizophyllan in water and DMSO (see Chapter II).

[b] See Chapter IV.

III-2-2. Viscometry

a. In water-DMSO Mixtures

Viscosities of samples S-144-2, S-166-3 and S-164-3 in water-DMSO mixtures at 25°C were measured using conventional viscometers of the Ubbelohde type. No correction for shear-rate effect was made. Solutions were prepared by mixing a given sample and a water-DMSO mixture of desired composition below 25°C. Densities

of water-DMSO mixtures at 25°C were measured as a function of water composition.

b. In Pure Water

Samples M-2 and U-1 in pure water at temperatures from 20 - 160°C were investigated using a capillary viscometer of the Ubbelohde type or a rolling-ball viscometer.[1-3] The former was used for determining the relative viscosity η_r and the latter the viscosity coefficient η.

A schematic diagram of the rolling-ball viscometer is shown in Figure III-1. The viscometer consists of

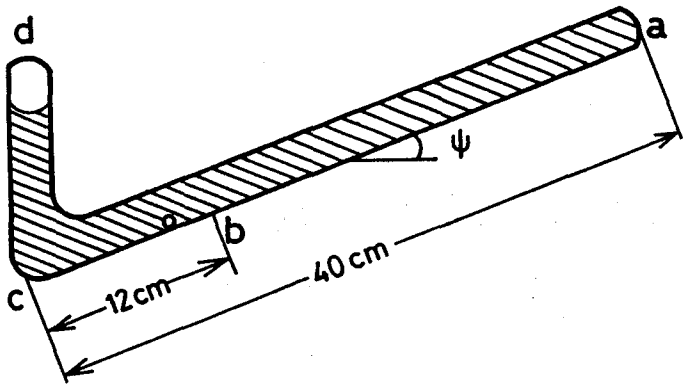

Figure III-1. Schematic diagram of a rolling-ball viscometer.

a glass tube of 7.60 ± 0.08 mm in inner diameter (Tokyo Garasu Kikai Co.) and a glass ball of 0.695 ± 0.003 mm in diameter, both made of SK soft glass with a density ρ_b of 2.50 g cm^{-3} (at 25°C) and a linear expansion coefficient α of 99 x 10^{-7} deg^{-1}.

The glass tube of the rolling ball viscometer was sealed after it was filled with a test solution; actually, air (about 0.5 cm^3) was left near one end (d) of the tube to prevent the tube from breaking. The viscometer was placed in an oil thermostat in such a way that the tube (a - c) made an angle ψ (see Figure III-1) of 15 - 25°. The ball was allowed to roll down from (b) to (c) points, and its velocity V was determined. The Raynold number defined by Hubbard's equation[1] was smaller than 4 for any aqueous solutions studied.

The viscosity coefficient η of a given test solution was evaluated from the equation[2,3]

$$\eta = \frac{C}{V} (\rho_b - \rho) \qquad (3\text{-}1)$$

where ρ is the solution density and C, a constant

dependent on ψ and the diameters of the tube and the ball. The viscometer was calibrated for different ψ with aqueous sucrose; C was independent of temperature ranging from 20 to 150°C and of the inner pressure generated in the glass tube. The values of ρ_b at different temperatures were calculated from ρ_b at 25°C and α, and those of ρ estimated from density data for pure water and the partial specific volume of schizophyllan in water at 25°C. The values of V ranged from 6.3×10^{-4} to 1.5 cm s^{-1}, and were reproducible within ± 3 % in the entire range of temperature studied. No correction for shear-rate effect was made.

III-2-3. Optical Rotatory Dispersion

ORD curves were taken for sample S-144-2 in water-DMSO mixtures at 25°C, using a JASCO ORD/UV-5 recording spectropolarimeter. Quartz cylindrical cells of 10 cm path length were used. The polymer mass concentration was fixed at about 0.5×10^{-2} g cm^{-3}.

III-2-4. Viscoelasticity

Dynamic shear moduli were measured on sample M-2 in water at 25°C using an autoviscometer of the coaxial

cylinder type (Iwamoto Seisakusyo Co.). The diameters of the inner and outer cylinders of the viscometer were 1.6 (or 2.0) and 2.2 cm, respectively, and the length of the inner cylinder was 7.1 cm. Lissajous' figures were recorded and analyzed by Markovitz's equation.[4]

III-3. Results and Discussion

III-3-1. Dissociation of the Triple Helix in Water-DMSO Mixtures

Figure III-2 illustrates the composition dependence of $[\eta]$ in water-DMSO mixtures at 25°C for three schizophyllan samples. With a decrease in water composition, $[\eta]$ stays essentially constant down to about 15 wt% water, decreases almost discontinuously around 13 %, and finally approaches the value in pure DMSO. This viscosity behavior implies that the triple helix of schizophyllan remains stable in the region of water composition about 15 wt%, but dissociates almost completely to three single chains with the addition of only a few percent DMSO to the mixture of 15 wt% water. Direct confirmation of this finding about the stability of the schizophyllan helix in water-DMSO mixtures was recently obtained by Sato et al.[5] from light scattering

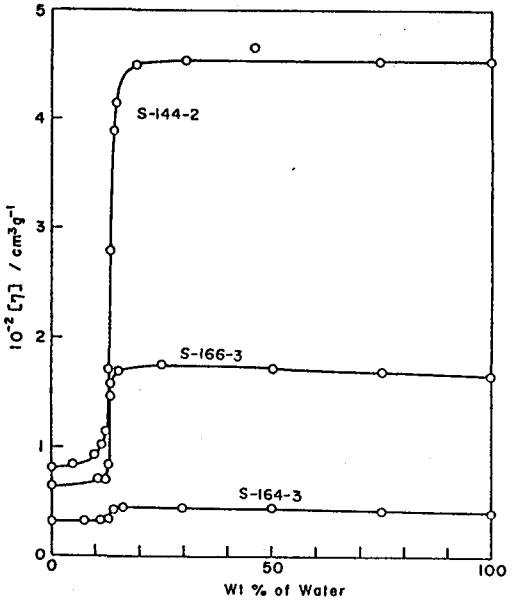

Figure III-2. Composition dependence of [η] for indicated schizophyllan samples in water-DMSO mixtures at 25°C.

measurements of M_w and $\langle S^2 \rangle$. It should be noted that the critical composition is essentially independent of helix length.

Figure III-3 shows ORD curves for sample S-144-2 in water-DMSO mixtures at 25°C. The curves for pure water and pure DMSO are distinctly different, but the transition from one to the other with water composition occurs continuously. As shown in the insert of Figure III-3, the specific rotation $[\alpha]_{589}$ at 589 nm wavelength

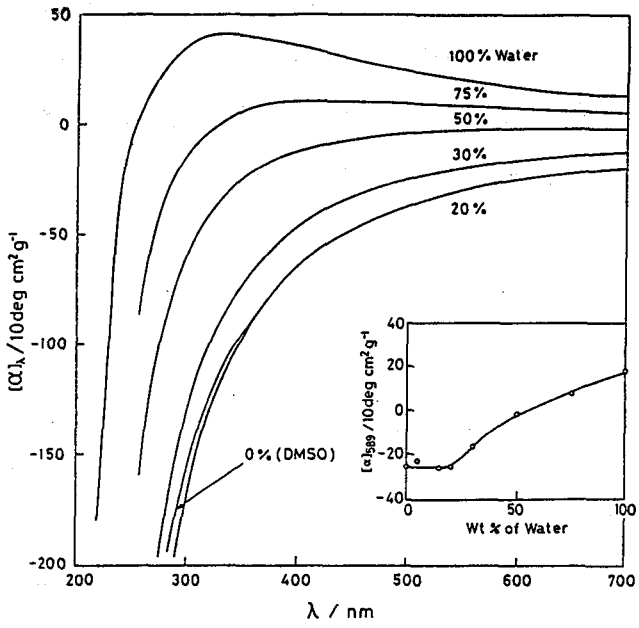

Figure III-3. ORD curves for sample S-144-2 in water-DMSO mixtures at 25°C. λ denotes the wavelength. The insert shows the composition dependence of $[\alpha]_{589}$ for the same sample in the mixed solvents.

is essentially constant (about -25 x 10 deg cm^2g^{-1}) in the composition range where $[\eta]$ was found to change discontinuously. This indicates no direct correspondence to exist between $[\eta]$ and $[\alpha]_{589}$ for schizophyllan in water-DMSO mixtures, in contradiction to the usual expectation[6,7] that specific rotation of a helix-forming polymer in dilute solution should directly reflect its

conformation. Instead it exemplifies a limited use of optical rotation data in exploring polysaccharide conformations in dilute solution.

III-3-2. Association in water-DMSO Mixtures

Figure III-4 shows the changes in $(\ln \eta_r)/c$ with time that occurred after DMSO solutions of sample S-144-2

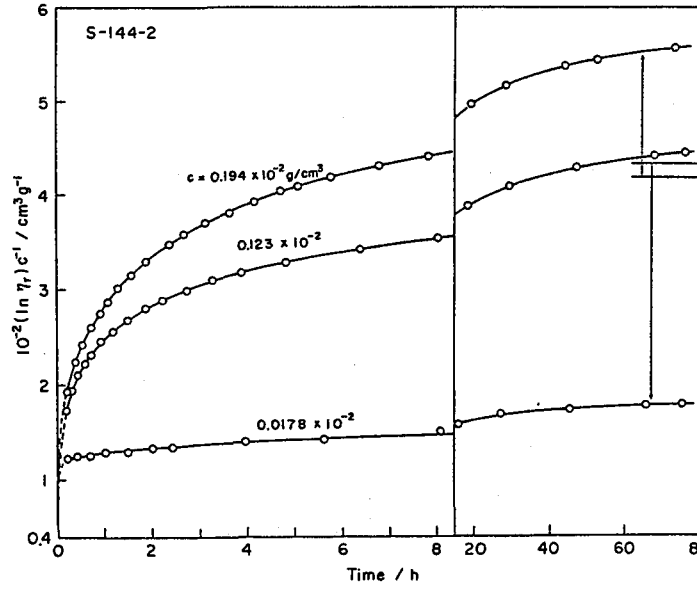

Figure III-4. Increases in $(\ln \eta_r)/c$ with time after dilution of DMSO solutions of sample S-144-2 with water to 10 wt% DMSO. The values of c indicate the polymer concentrations in 10 wt% DMSO. See the text for the implication of the two segments.

with different c had been `diluted' with water to 10 wt% DMSO. Note that schizophyllan maintains the triple helical structure when dissolved directly in a mixture of this composition. The horizontal segments in Figure III-4 represent the values of $(\ln \eta_r)/c$ which were obtained at the indicated c when sample S-144-2 was dissolved directly in a mixture of 10 wt% DMSO. The curve for the lowest c levels off at a value far below the segment for this concentration, while that for the highest c exceeds the level of the corresponding segment after 7 h and appears to rise indefinitely. These data suggest that once `denatured' to single chains in pure DMSO, the intact triple-helical trimer of schizophyllan can no longer be restored by addition of water to the solution.

As mentioned in Chapter II, the schizophyllan triple helix is stabilized by interchain hydrogen bonds. Thus, when the solvent is changed from pure DMSO to 10 wt% DMSO by addition of water, the hydroxyl groups of randomly coiled schizophyllan chains should have chances to form interchain hydrogen bonds. However, it must be extremely unlikely that three non-specific chains in dilute solution are hydrogen bonded to reform a triple

helix. It is far more likely that two, three, or more chains are randomly hydrogen bonded to form dimers, trimers, and higher aggregates. As c is increased, there are more chances for schizophyllan chains to meet one another, and hence larger aggregates are formed, leading to a viscosity higher than that expected for a rodlike triple helix.

These considerations are consistent with the viscosity behavior observed in Figure III-4. In this connection, we remark that collagen heat-denatured at high concentrations does not restore the intact triple helical structure but forms large aggregates when the aqueous solution is cooled.[8-10] The kinetic curves obtained by Engel[8] in a renaturation experiment on collagen are similar to those for schizophyllan in Figure III-4.

III-3-3. Melting of the Triple Helix in Pure Water

Figure III-5 shows the variations of η with temperature observed when pure water solutions of samples M-2 and U-1 were heated from 20 to about 160°C and cooled to 20°C, at a rate of 0.50 ± 0.03 deg min^{-1}. It can be seen that on heating, η for either sample decreases almost linearly up to 130°C, very sharply at about 135°C,

and again linearly above 140°C. The linear decreases in η below 130°C and above 140°C may be described by a Arrhenius type temperature dependence, but the sharp decrease in η at T ∿ 135°C cannot. On the other hand,

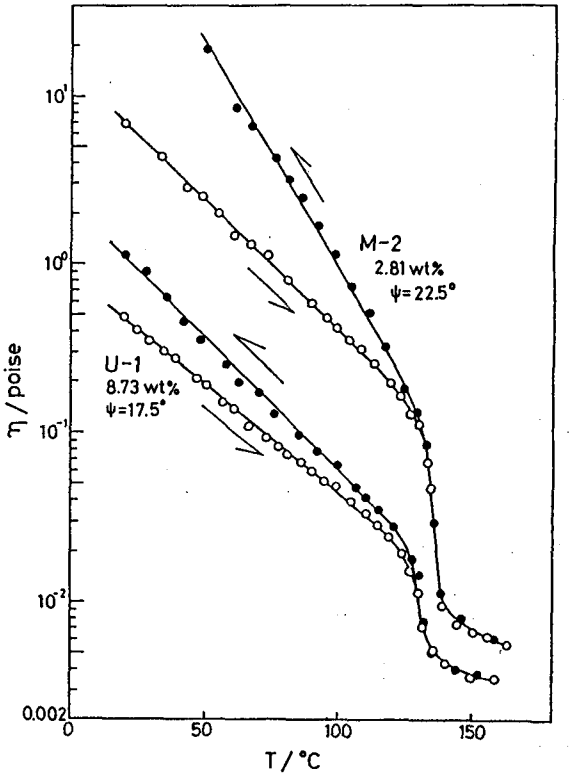

Figure III-5. Temperature dependence of η for schizophyllan samples M-2 and U-1 in pure water. Unfilled circles, heating; filled circles, cooling; rate of heating or cooling, 0.50 ± 0.03 deg min^{-1}.

on cooling, η restores following the same curve as that on heating down to about 130°C, and deviates upward progressively from it below 130°C. Thus, schizophyllan in water undergoes an irreversible, thermal change at $T \sim 135$°C.

Similar measurements on the same samples at different polymer concentrations (0.8 - 12 wt%) gave substantially the same results as above. Hence, we can conclude the thermal change in schizophyllan to occur virtually independently of the concentration and molecular weight of the polymer.

Figure III-6 shows the changes in $(\ln \eta_r)/c$ at 25°C with time that occurred after an aqueous solution of sample M-2 with $c = 0.106 \times 10^{-2}$ g cm^{-3} was preheated at 150°C for different periods Δt_p (1 - 10 min) and cooled to 25°C. The curves for $\Delta t_p \leqq 4$ min depend strongly on Δt_p, whereas those for $\Delta t_p \geqq 6$ min almost superimpose. The initial $(\ln \eta_r)/c$ values (about 1×10^2 cm^{3}g^{-1}) for the latter group of curves are comparable to $[\eta]$ of the same sample in DMSO at 25°C. This indicates that schizophyllan is dispersed in pure water at 150°C as single random coils.

Similar measurements on aqueous solutions of sample

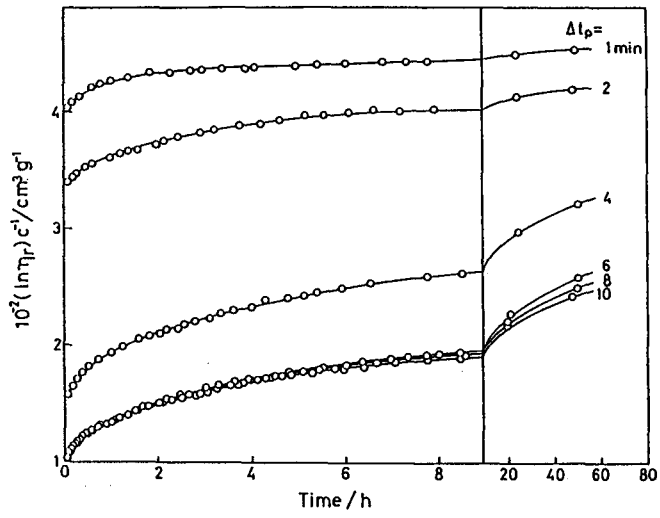

Figure III-6. Increases in $(\ln \eta_r)/c$ at 25°C with time that occurred after aqueous schizophyllan solutions preheated at 150°C for periods Δt_p of 1 - 10 min were cooled to 25°C. Sample, M-2; c, 0.106 x 10^{-2} g cm^{-3}.

M-2 preheated at 120°C showed that $(\ln \eta_r)/c$ are virtually independent of Δt_p, ranging from 4.2 x 10^2 to 4.4 x $10^2 cm^3 g^{-1}$. The close agreement of these $(\ln \eta_r)/c$ values with $[\eta]$ for the intact triple helix of the same sample in water at 25°C (see Table III-1) indicates that the majority of the schizophyllan chains in water maintained the triple helical structure up to as high a temperature as 120°C.

Now that the major conformations of schizophyllan

in water at 120 and 150°C have been found to be triple helix and single random coil, respectively, the sharp drop in η at about 135°C in Figure III-5 may be interpreted as due to the melting of triple helices to single chains.

Figure III-7 shows the time dependence of $(\ln \eta_r)/c$ at 25°C for aqueous solutions of sample M-2 preheated at 150°C for 10 min. The curves resemble those observed for water-DMSO mixtures in Figure III-4 and show that

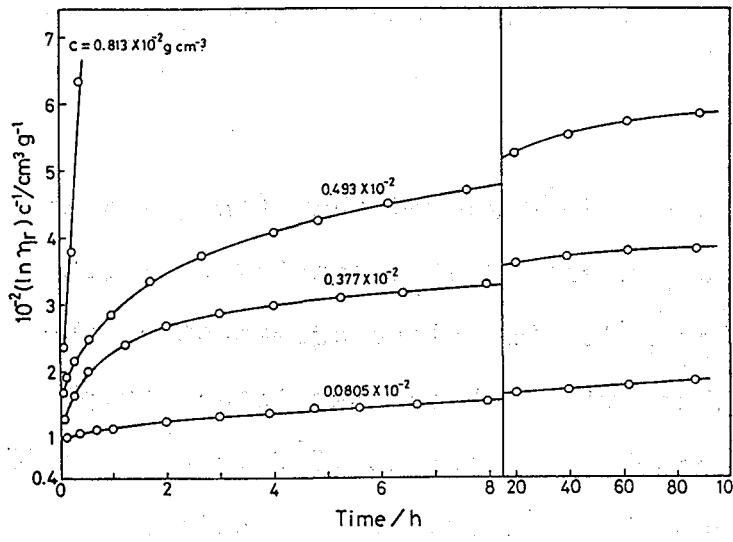

Figure III-7. Time dependence of $(\ln \eta_r)/c$ at 25°C for aqueous solutions of sample M-2 preheated at 150°C for 10 min.

schizophyllan single chains at 150°C in pure water aggregate on cooling. The curve for the highest c rises very abruptly. The solution of this c lost almost completely its fluidity in about 30 min, and gel formation was suspected. Similar behavior was observed for aqueous M-2 solutions after the thermal curves of η in Figure III-5 had been determined.

Figure III-8 illustrates the frequency dependence of dynamic strage modulus G' and dynamic loss modulus G" at 25°C for a 3.23 wt% aqueous M-2 solution. The filled circles represent the data obtained 5 h after a test solution was preheated at 150°C for 10 min, while the unfilled ones refer to a non-preheated solution. The curves of G' and G" for the two solutions are distinctly different. For the non-preheated solution, G" is larger than G' throughout the entire frequency range studied, and both exhibit behavior typical of a molecularly dispersed system. On the other hand, the two moduli for the preheated solution show behavior characteristic of gels; G' is larger than G", and both moduli little depend on frequency. From these data it may be concluded that schizophyllan forms a gel when its aqueous solution with a concentration higher than a

certain value is heated to about 150°C and cooled to room temperature.

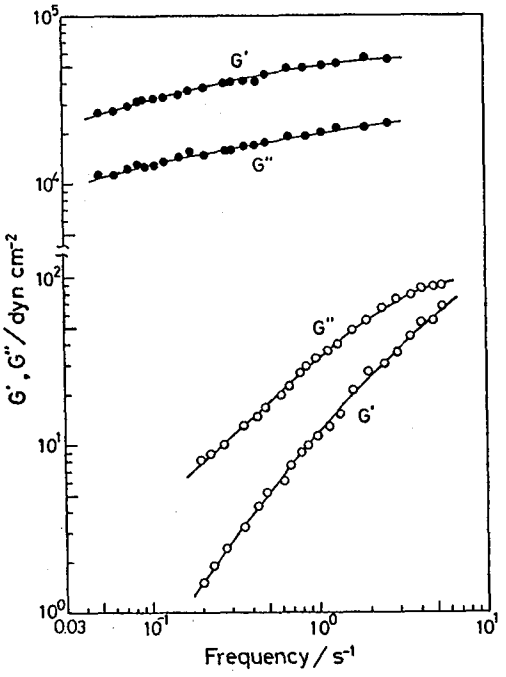

Figure III-8. Frequency dependence of G' and G" at 25°C for an aqueous M-2 solution of 3.23 wt%. (○) and (●), before and after preheating at 150°C for 10 min, respectively.

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CHAPTER IV

CORRELATION BETWEEN THE ANTITUMOR ACTIVITY OF SCHIZOPHYLLAN AND ITS TRIPLE-HELICAL CONFORMATION IN DILUTE AQUEOUS SOLUTION

IV-1. Introduction

An aqueous solution of native schizophyllan has a host-mediated antitumor activity against Sarcoma 180,[1] a tumor of mice. Recently, Tabata et al.[2] showed that fragmentation of native schizophyllan by sonication does not impair the antitumor potency of the original sample, if the molecular weights of sonicated samples in water, estimated from the viscosity-molecular weight relation established in this work (see Figure II-8 in Chapter II), are higher than about 2.5×10^5. In Chapter II, we found that both native and sonicated samples of schizophyllan ranging in molecular weight from 10^5 to 6×10^6 dissolve in water as trimers having a rigid triple-helical structure.

In this chapter, we are concerned with the question whether the antitumor activity and triple-helical structure of schizophyllan are still maintained when the

molecular weight is decreased below 10^5 by extensive sonication. Thus, bioassay, sedimentation equilibrium, viscosity, and gel-filtration experiments were performed on eight extensively sonicated samples. The results obtained are discussed in relation to Chihara's hypothesis[3] mentioned in Chapter I.

IV-2. Experimental

IV-2-1. Samples

From sonicated schizophyllan samples stored for clinical use at Taito Co., one with a molecular weight (in water) of 4.5×10^5 was chosen for the present study. Its aqueous solution (about 4 % polymer) was exposed to 19.5 kHz sonic irradiation (Kaijo Denki, Model TA-6280N) for about 500 h, with pasteurization at suitable intervals of time. The jacket of the sonication vessel was kept below 20°C by circulating water at 10°C. The sonicated solution was passed through an 0.3 μm Millipore filter after being deionized with ion-exchange resins (Nippon Orugano, IRA 402 and IR 120B). A schizophyllan sample was recovered from it and separated into 20 parts by repeating fractional

precipitation with water as the solvent and acetone as the precipitant. Eight fractions designated below as U-1, U-4, U-6, U-11, U-15, U-16, U-17, and U-18 were chosen, and first freeze-dried from aqueous solutions and then vacuum-dried overnight. Methylation analysis and enzymic hydrolysis with exo-β-1,3-D-glucanase showed that all these fractions consisted of the same repeating units as that shown in Figure I-1.

IV-2-2. Assay of Antitumor Activity

Sarcoma 180 ascites (0.1 cm^3; about 2 x 10^6 cancerous cells) was implanted subcutaneously into the groins of 30 ICR-JCL mice (Clea Japan Co.) weighing 20 - 25 g. After 24 h, physiological saline dissolving a given schizophyllan sample (polymer concentration 0.5 wt%) was intramuscularly injected into 10 of these mice at an optimum dose[4,5] of 10 mg schizophyllan per kg of mouse. 31 days after tumor implantation, all the mice were killed and dissected, and ξ defined by eq 1-1 and the complete regression were evaluated by w_c and w_t (see Section I-3 in Chapter I). The value of w_c was 2.57 g with a standard deviation (S.D.) of 2.08 g.

IV-2-3. Sedimentation Equilibrium

Sedimentation equilibrium measurements were made on samples U-1, U-4, U-6, U-11, U-15, U-16, U-17, and U-18 in water and on samples U-6, U-15, U-17, and U-18 in DMSO at 25°C, using a Beckman Model E ultracentrifuge. A Kel-F 12 mm double-sector cell was used, with liquid columns of 1.2 - 2.5 mm. Data were analyzed by the method described in Chapter II.

The $\partial n/\partial c$ for schizophyllan in water at 25°C was 0.145 cm^3g^{-1} for 436 nm wavelength and 0.143 cm^3g^{-1} for 546 nm, independent of molecular weight, when determined with samples U-4, U-10, U-16, U-17, and U-18. These values agreed with those determined in Chapter II with higher molecular weight samples. For $\partial n/\partial c$ in DMSO and for $\bar{v}$ in water and DMSO the values given in Section II-2-2 of Chapter II were used.

IV-2-4. Gel-Filtration Chromatography

Five schizophyllan samples U-1, U-4, U-11, U-16, and U-18 were dissolved in 0.05 M sodium acetate buffer and investigated by gel-filtration chromatography with a Sephadex G-100 column 2.5 cm wide and 45 cm long. The polymer concentration of the loaded solution was

0.15 wt%, and the flow rate 0.11 $cm^3 min^{-1}$. Each elute (80 cm^3) was separated into 40 fractions, and their sugar content was determined by the phenol-sulfuric acid method.[6] The gel-filtration chromatograms obtained were assumed to be the same as those in pure water, since virtually no viscosity difference was detected between pure water solutions and acetate buffer solutions.

IV-2-5. Viscometry

Viscosities of the samples in water and DMSO at 25°C were measured using capillary viscometers of the Ubbelohde suspended-level type. In converting the measured flow times to relative viscosities, correction for the solution density was made.

IV-3. Results and Discussion

IV-3-1. Antitumor Activity and Molecular Weight

Bioassay data are summarized in Table IV-1, along with those from sedimentation equilibrium measurements. The values of ξ and the complete regression for samples U-1 and U-4 indicate potent antitumor activities against

Table IV-1. Bioassay and sedimentation equilibrium data on schizophyllan samples

Sample	w_t (±S.D.)/g	ξ	Complete regression	In water		In DMSO		M_w(in water) / M_w(in DMSO)
				$10^{-4}M_w$	10^4A_2[a]	$10^{-4}M_w$	10^4A_2[a]	
U-1	0.02 (±0.03)	0.992	9/10	13.4	2.6	4.7[b]	—	2.9
U-4	0.01 (±0.02)	0.996	9/10	9.50	3.9	3.6[b]	—	2.6
U-6	0.24 (±0.68)	0.91	9/10	7.40	2.3	3.08	10	2.4
U-11	0.53 (±0.85)	0.79	4/10	5.54	1.0	2.5[b]	—	2.2
U-15	2.12 (±2.14)	0.18	3/10	4.08	—	1.96	15	2.1
U-16	2.14 (±1.73)	0.17	2/10	2.51	—	1.35[c]	21[c]	1.86
U-17	—	—	—	0.769	—	0.749	27	1.03
U-18	3.27 (±2.06)	-0.27	0/9[d]	0.503	—	0.493	28	1.02

[a] In units of $cm^3 mol\ g^{-2}$.

[b] Evaluated from [η] using the relation between [η] and M_w for schizophyllan in DMSO (see Chapter II).

[c] Taken from ref. 7.

[d] One mouse died of the ascites cancer 20 days after tumor implantation.

Sarcoma 180 ascites. These values are comparable to those determined by Tabata et al.[2] for three higher-molecular weight samples of schizophyllan, indicating that when M_w(in water) is above about 9×10^4, the antitumor potency of aqueous schizophyllan is independent of molecular weight. However, as M_w(in water) decreases below 6×10^4, ξ decreases abruptly and even becomes negative at M_w(in water) $\sim 5 \times 10^3$. The complete regression also diminishes sharply in the same molecular weight region. Thus, we see that schizophyllan almost loses its antitumor activity when sonicated to fragments of M_w(in water) of the order of 10^4.

IV-3-2. Molecular Species in Aqueous Solution

The ninth column of Table IV-1 indicates that the molecular weight ratio Γ [$\equiv M_w$(in water)/M_w(in DMSO)] is fairly close to 3 for the two highest-molecular weight samples U-1 and U-4, but decreases monotonically with decreasing M_w(in water) and approaches unity at M_w(in water) $\sim 10^4$. This behavior shows that the predominant species of samples U-1 and U-4 in aqueous solution are a trimer, while those of the two lowest-

molecular weight samples U-17 and U-18 are a monomer.

The gel-filtration chromatograms of some schizophyllan samples are shown in Figure IV-1. The curves for the two highest-molecular weight samples U-1 and U-4 are essentially single peaked, but that for sample U-4 exhibits a broad tail in the region of large elution counts. As the molecular weight is lowered, this tail shows up a second peak, which eventually surpasses the first peak. Thus, except for U-1, the schizophyllan samples examined consisted of at least two species of different molecular weights, and the fraction of the lower-molecular weight species increased with decreasing M_w of the sample. If this is combined with the above finding that the predominant species of samples U-1 and U-4 in water are a trimer and that of sample U-18 is a monomer, the first and second peaks in Figure IV-1 may be assigned to the trimer and the monomer, respectively.

The weight fraction f of trimers in a given aqueous solution may be calculated from the relation

$$f = (\Gamma - 1)/2 \qquad (4\text{-}1)$$

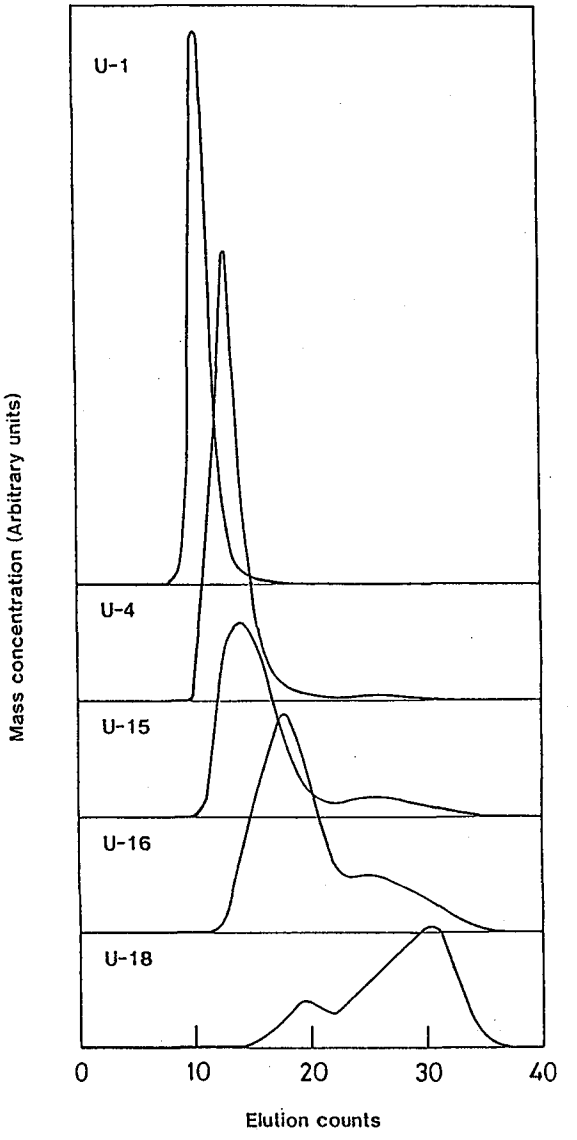

Figure IV-1. Gel-filtration chromatograms of schizophyllan samples in 0.05 M aqueous sodium acetate.

provided that M_w of the trimer species in the solution is three times that of the monomer species. The fraction f may also be estimated from the gel-filtration chromatograms in Figure IV-1 if they can be resolved uniquely to the chromatograms of the two species. For example, a reasonable resolution of the curve for sample U-18 gives f a value between 0.1 and 0.2. However, this is much larger than 0.01 evaluated from Γ using eq 4-1. A similar discrepancy was found for sample U-4, for which the values of f from Γ and the chromatogram were 0.8 and 0.9, respectively. At present, no reasonable explanation can be made for these discrepancies. Here, the values of f from Γ are considered reliable and used for the subsequent data analysis.

Figure IV-2 shows double-logarithmic plots of $[\eta]$ vs. M_w for schizophyllan in water and DMSO at 25°C. Here, the smaller circles indicate the data obtained for higher-molecular weight samples in Chapter II, and the dashed line represents the theoretical values calculated for the schizophyllan triple helix (pitch per main chain residue = 0.30 nm and diameter = 2.6 nm, both determined in Chapter II) using the theory of

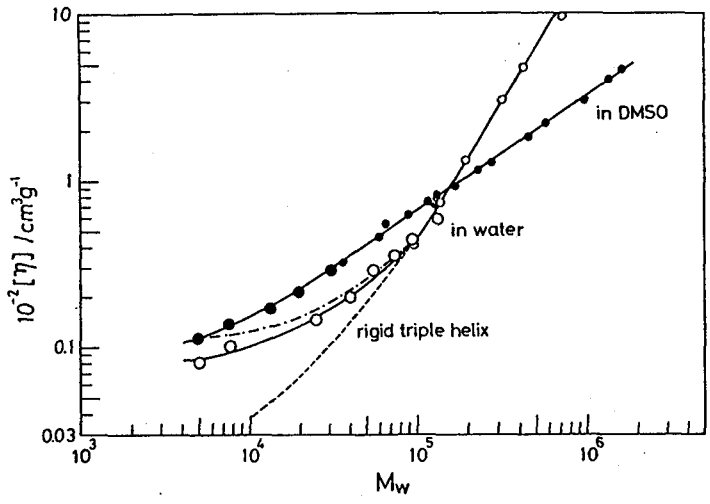

Figure IV-2. Double-logarithmic plots of [η] vs. M_w for schizophyllan in water and DMSO at 25°C. The smaller circles indicate the data obtained for higher-molecular weight samples in Chapter II, and the dashed and dot-dash lines represent, respectively, the calculated values for the schizophyllan triple helix with a pitch (per residue) of 0.30 nm and a diameter of 2.6 nm and for polymer mixtures consisting of triple helices and single randomly coiled chains.

Yoshizaki and Yamakawa[8] for straight rods. This line approximately fits the data points for the three highest-molecular weight samples U-1, U-4, and U-6 in

water, indicating that the trimers of these samples in water have the same rigid triple-helical structure as that found in Chapter II. Another significant fact is that $[\eta]$ for aqueous solutions begins to deviate upward from the dashed line at $M_w \sim 7 \times 10^4$ and the deviation is more appreciable for lower M_w.

The intrinsic viscosity of a polymer mixture of trimers and monomers is given by

$$[\eta] = f[\eta]_3 + (1 - f)[\eta]_1 \qquad (4\text{-}2)$$

regardless of the chain length distributions in the two species. Here, $[\eta]_3$ and $[\eta]_1$ denote the intrinsic viscosities of the trimer and monomer, respectively. If it is assumed that the trimers and monomers of schizophyllan in water are, respectively, rigid triple helices and the same random coils* as those in DMSO (see Chapter II), then $[\eta]$ of a given schizophyllan

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* Since the molecular chains of our samples are very short [for example, about 46 in main chain residues for M_w(in DMSO) $\sim 10^4$], a difference in excluded-volume effect between water and DMSO solutions may be ignored.

sample in water may be estimated from eq 4-2 and the dashed and DMSO lines in Figure IV-2; for a given value of M_w(in DMSO), $[\eta]$ interpolated at $3M_w$(in DMSO) from the dashed line may be taken as $[\eta]_3$.

The values of $[\eta]$ thus calculated are shown by a dot-dash line in Figure IV-2. For M_w between 7×10^4 and 10^4, the line is quite close to the experimental points for aqueous solutions, confirming that the schizophyllan trimer in this molecular weight range is rigid triple-helical, while the monomer in water is randomly coiled as it is in DMSO.

IV-3-3. Effects of Extensive Sonication

The viscosity and chromatographic data presented above have shown that our sonicated schizophyllan in water is a mixture of triple helices and single chains when its M_w(in water) is lower than about 10^5 and that the relative amount of single chains increases with decreasing M_w(in water). It is reasonable to consider that dissociation of triple helices occurred not at the time of dissolving a given sample in water for either viscosity or chromatographic measurement but during sonication. It is likely that extensive sonication as

employed in this work weakens interchain hydrogen bonds of triple helices because it generates localized heat and stress. Dissociation to single chains eventually occurs when the fragmented helices reach a molecular weight lower than a certain value. From our experimental data such a critical molecular weight (in water) is estimated to be about 10^5. As M_w(in water) is lowered further by more extensive sonication, more single chains should appear in the solution, as actually found by our experiment.

IV-3-4. Correlation between Antitumor Activity and Helix Fraction

As can be seen from Table I-1 in Chapter I, some β-1,3-D-glucans with or without β-1,6-D-glucose side chains have host-mediated antitumor activities against Sarcoma 180, but others do not. A subject in recent pharmacological studies is concerned with a primary factor for antitumor activities of glucans. Chihara[3] proposed for it a certain ordered structure of a glucan in aqueous solution, though he did not specify the 'ordered' structure (see Chapter I).

Our bioassay has shown that schizophyllan loses

antitumor potency when M_w(in water) is lowered to about 10^4. This critical molecular weight is comparable to those ($5 - 8 \times 10^3$) found by Sasaki et al.[9] for curdlan and lentinan. These authors and also Saito et al.[10] concluded from bioassay and spectroscopic results that the loss of the antitumor potency of lentinan is associated with a certain order-disorder conformation change of the glucan accompanying a decrease in molecular weight. Our study has clearly shown that the predominant species of schizophyllan in water changes from a trimer of rigid triple-helical structure to a single separated chain in the range of molecular weight from 10^5 to 10^4. This is consistent with Chihara's proposal, and suggests that the triple helix is the ordered structure invoked by Chihara as the primary factor for the antitumor activity of β-1,3-D-glucan.

In Figure IV-3, the values of ξ given in Table IV-1 are plotted against the weight fraction of triple helices evaluated from eq 4-1. It may be concluded from the smooth curve fitting the data points that the loss of the antitumor potency of schizophyllan in water is related to the decrease in the amount of triple helices relative to that of single separated chains in

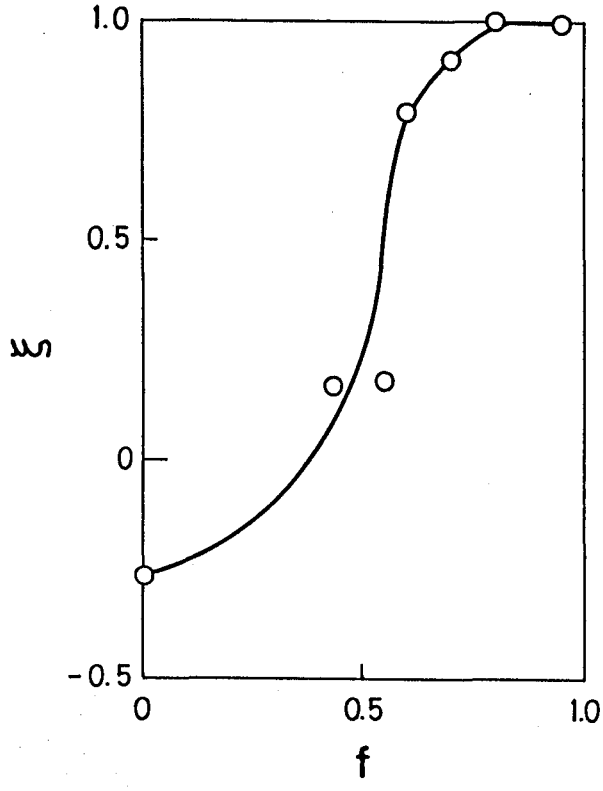

Figure IV-3. Relation between tumor inhibition ratio and the weight fraction f of triple helices for schizophyllan.

the solution and that an aqueous schizophyllan containing less than about 50 % triple helices has virtually no potent antitumor activity.

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2. ref. 9 in Chapter I.

3. ref. 16 in Chapter I.

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8. T. Yoshizaki and H. Yamakawa, Dynamics of Spheroid-Cylindrical Molecules in Dilute Solution, J. Chem.

Phys., 72, 57 - 69 (1980).

9. ref. 12 and 19 in Chapter I.

10. ref. 20 in Chapter I.

CHAPTER V

TRIPLE HELIX AND RANDOM COIL OF POLYTRAN SCLEROGLUCAN IN DILUTE SOLUTION

V-1. Introduction

Polytran scleroglucan is a capsular β-1,3-D-glucan elaborated by the fungus Sclerotium rolfsii.[1] Although it is usually considered the same polysaccharide as schizophyllan, its chemical structure is yet undetermined.

This chapter deals with the question whether or not Polytran scleroglucan is chemically identical with schizophyllan and whether these two polysaccharides show the same dimensional and hydrodynamic behavior in dilute solution. For the former, we applied the same methods of chemical analysis as those established for schizophyllan. For the latter, we determined M_w, $\langle S^2 \rangle^{1/2}$, s_0, and $[\eta]$ of Polytran samples in water and DMSO. In the actual measurements on aqueous solutions, we added 0.01 N sodium hydroxide (NaOH) to water, as Kashiwagi et al.[2] did in their recent study on schizophyllan. These authors showed that addition of

0.01 N NaOH enhances the solubility of schizophyllan in water without impairing the triple helical structure.

V-2. Experimental

V-2-1. Samples

A scleroglucan sample (Ceca S.A. Polytran R), supplied by Mitsui-Bussan Co., was purified by the method established for schizophyllan (see Chapter II). A purified sample (designated below as N) dissolved in water gave a perfectly transparent solution. Part of sample N was sonicated by the method described in Chapter II to obtain fragments of different chain lengths. The fragmented samples designated below as H, F, D, I, S, C, B, J, and E, and sample N were each divided into three to seven parts by fractional precipitation with water as the solvent and acetone as the precipitant. Appropriate middle fractions, designated below as N-2, H-1, F-1, D-3, I-1, I-3, S-2, C-2, C-3, C-5, B-3, B-4, J-1, J-3, and E-4, were selected, reprecipitated from aqueous solutions into acetone, and freeze-dried from aqueous solutions.

These fractions were dried overnight in vacuo before use.

V-2-2. Chemical or Biochemical Analysis

Chemical or biochemical analysis was made on two samples: an unsonicated sample N-2 and a sonicated sample S-2.

a. Analysis of Component Sugars

Each of the two samples N-2 and S-2 (10 mg) was hydrolyzed with 6 N sulfuric acid (1 cm^3) for 1 h at 120°C, and the hydrolysate was made neutral with barium carbonate. The turbid neutral solution was centrifuged, and the supernatant was passed through a column of Amberlite IR-120B and a membrane filter. After the elute was concentrated and brought to a pH higher than 8 by the addition of 2 N ammonium hydroxide, the sugars in the elute were reduced with sodium borohydride (50 mg) at room temperature. The excess borohydride was decomposed by adding acetic acid. To remove the residual borate, methanol (2 cm^3) was added to the reaction mixture and allowed to evaporate at 40°C under reduced pressure. This procedure was repeated five

times to ensure the complete removal of borate. The resulting alditols were acetylated by heating with a 1 : 1 pyridine-acetic anhydride mixture (0.5 cm^3) for 2 h at 100°C.

The products were investigated by gas chromatography at 190°C using a 2-m column of 3 % ECNSS M-Gaschrom Q. The results indicated that the two Polytran samples consisted only of D-glucoses.

b. Methylation

The samples S-2 and N-2 were methylated by the method of Hakomori.[3] Each (20 mg) was dissolved in 2 cm^3 DMSO under a nitrogen atmosphere, and the resulting solution was treated with methylsulfinyl carbanion (0.5 cm^3) for 4 h at room temperature and then with methyl iodide (1.5 cm^3) for 2 h at 25°C. The solution was diluted with 10 cm^3 water and dialyzed against water. The whole methylation procedure was repeated until infrared absorption peaks characteristic of the hydroxyl groups of D-glucans disappeared.

The fully methylated glucans (5 mg) were hydrolyzed with 90 % formic acid (0.4 cm^3) for 12 h at 100°C and heated with 2 M trifluoroacetic acid (0.5 cm^3) for 7 h

at the same temperature. The methylated sugars were acetylated in the same way as described above. The products were examined by gas chromatography.

c. Periodate Oxidation and Mild Smith Degradation

Each Polytran sample (80 mg) was oxidized with 0.01 M sodium metaperiodate (50 mg) at 5°C in the dark. The consumption of periodate and the production of formic acid were traced as functions of time by the Fleury-Lange method[4] and by titration with 0.01 N NaOH, respectively. After completion of the oxidation, ethylene glycol (10 cm^3) was added and the mixture was dialyzed against water for 24 h at 5°C.

The oxidized glucans were reduced with sodium borohydride, and the resulting glucan-polyalcohols were slowly hydrolyzed with 0.1 N sulfuric acid at room tempereture (mild Smith degradation). The Smith-degraded glucans, which should have no β-1,6-linkage, were water-insoluble. They were collected by centrifugation, washed thoroughly with water, dried in vacuo at 40°C, and used for subsequent experiments.

d. Hydrolysis with Exo-β-1,3-D-Glucanase

Each Smith-degraded glucan (20 mg) was dispersed in 1.5 cm^3 of a McIlvaine buffer (pH 4) at 50°C and hydrolyzed with an enzyme exo-β-1,3-D-glucanase prepared from a culture of Basidiomycetes QM-806.[5] The reducing power of the solution was determined as a function of time by the Somogyi-Nelson method.[6] The original samples N-2 and S-2 were similarly hydrolyzed with exo-β-1,3-D-glucanase. All the hydrolysates were investigated by paper chromatography with Whatman No. 50 paper and 1-buthanol + 2-propanol + water (3 : 12 : 4) as the developing solvent.

V-2-3. Infrared (IR) Spectroscopy

IR spectra of samples N-2 and S-2 were obtained at room temperature on a Hitachi Grating Infrared Spectrophotometer. The test specimens were prepared by the KBr method.[7]

V-2-4. Light Scattering Photometry

a. Measurement

Intensities of light scattered from Polytran scleroglucan in 0.01 N aqueous NaOH and DMSO at 25°C

were measured on a Fica 50 automatic light scattering photometer in an angular range from 22.5 to 150°. Vertically polarized incident light of 436 nm or 546 nm wavelength was used.

b. Calibration

The Fica 50 photogoniometer was optically aligned in such a way that the normalized intensity $(I_{\theta,U_v}/I_{90,U_v})$ x sin θ did not deviate more than ± 0.005 from unity in the range of scattering angle from 22.5 to 150° when vertically polarized light of 436 nm was incident to an aqueous solution of fluorescein (concentration ∿ 3 x 10^{-7} g cm^{-3}). Here, I_{θ,U_v} and I_{90,U_v} denote the scattering intensities measured for vertically polarized incident light with no analyzer at scattering angles θ and 90°, respectively.

Benzene of 25°C was used to calibrate the photogoniometer. The measured values of $(I_{\theta,U_v}/I_{90,U_v})$ x sin θ shown in Figure V-1 are constant within ± 1 % in the angular range studied, indicating the absence of stray light as well as the success in purifying the liquid. The instrument constant Ψ was determined by use of the equation

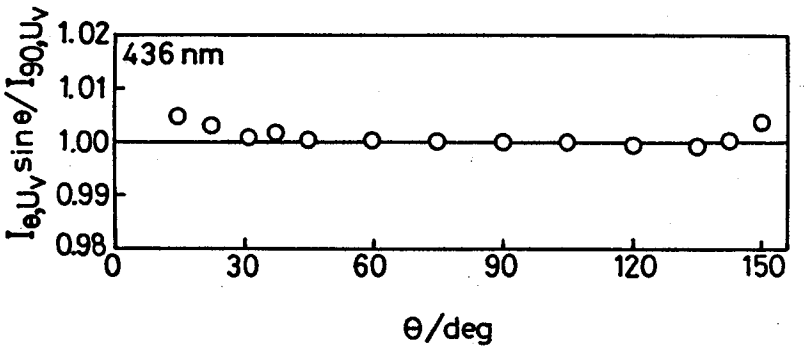

Figure V-1. Normalized intensities $(I_{\theta,U_v}/I_{90,U_v})\sin\theta$ for pure benzene at 25°C.

$$\Psi = \frac{2R_{90,U_u}}{I_{90,U_v} n_b^2 (1 + \rho_u)} \qquad (5\text{-}1)$$

where R_{90,U_u} is the 90° Rayleigh ratio of benzene for unpolarized light and n_b, the refractive index of benzene. The literature values[8] of 46.5×10^{-6} cm^{-1} for 436 nm and 16.1×10^{-6} cm^{-1} for 546 nm were used for R_{90,U_u}; and the depolarization ratio ρ_u of 25°C benzene was estimated to be 0.41_0 for 436 nm and 0.40_6 for 546 nm using the equation derived by Rubingh and Yu:[9]

$$\frac{I_{\theta,U_u}}{I_{90,U_u}} \sin\theta = \frac{1 - \rho_u}{1 + \rho_u} \cos^2\theta + 1 \qquad (5\text{-}2)$$

Here, I_{θ,U_u} is the scattering intensity at θ for unpolarized light. We note that our ρ_u values are in good agreement with the literature data.[10,11]

Reduced scattering intensities R_θ ($\equiv R_{\theta,U_v}$) were calculated from measured values of I_{θ,U_v} according to the equation

$$R_\theta = \Psi I_{\theta,U_v} n_0^2 \tag{5-3}$$

where n_0 denotes the refractive index of the solvent.

c. Data Analysis

The intensity of light scattered from a dilute polymer solution is expressed by[12]

$$\frac{Kc}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 Q(\theta) c + \cdots \tag{5-4}$$

with

$$P(\theta)^{-1} = 1 + \frac{16\pi^2 n_0^2}{3\lambda^2} \langle S^2 \rangle \sin^2(\theta/2) + \cdots \tag{5-5}$$

$$K = \frac{2\pi^2 n_0^2 (\partial n/\partial c)^2}{N_A \lambda^4} \tag{5-6}$$

Here, $P(\theta)$ and $Q(\theta)$ are the intramolecular and intermolecular scattering functions, respectively, and λ the wavelength of incident light in vacuum. At infinite dilution eq 5-4 gives

$$\left(\frac{Kc}{R_\theta}\right)_{c=0} = \frac{1}{M_w}\left[1 + \frac{16\pi^2 n_0^2}{3\lambda^2}\langle S^2\rangle \sin^2(\theta/2) + \cdots\right] \qquad (5\text{-}7)$$

At the limit of zero scattering angle, both $P(\theta)$ and $Q(\theta)$ approach unity, so that eq 5-4 gives

$$\frac{Kc}{R_0} = \frac{1}{M_w} + 2A_2 c + \cdots \qquad (5\text{-}8)$$

$$\left(\frac{Kc}{R_0}\right)^{1/2} = \left(\frac{1}{M_w}\right)^{1/2}(1 + A_2 M_w c + \cdots) \qquad (5\text{-}9)$$

In this work, the measured values of Kc/R_θ as a function of c and θ were extrapolated to zero scattering angle and infinite dilution by use of the plots of Kc/R_θ vs. $\sin^2(\theta/2)$ and Kc/R_θ vs. c, respectively; for DMSO solutions the plot of $(Kc/R_\theta)^{1/2}$ vs. c was used to determine M_w and A_2, since Kc/R_θ data plotted against c exhibited upward curvatures (see

Figure V-7).

d. Optical Anisotropy

The optical anisotropies of samples I-1, C-2, and E-4 in 0.01 N NaOH at 25°C were investigated at a fixed scattering angle of 90° with a polarizer fixed in the vertical direction. The scattered intensity measured with an analyzer set in the horizontal direction did not exceed 0.7 % of the intensity measured with the analyzer in the vertical direction. Furthermore, when the light scattering envelopes of sample E-4 for vertically polarized incident light and unpolarized incident light were compared, no difference was detected. Thus, Polytran scleroglucan in 0.01 N NaOH was concluded to be optically isotropic.

e. Optical Purification

Aqueous solutions of Polytran scleroglucan containing 0.01 N NaOH were made optically clean by filtration through a membrane filter and by 4 h centrifugation at about 4×10^4 gravities in a Sorvall RC2-B centrifuge. Use was made of Millipore's SSWP 047 00 for samples N-2 and H-1, BSWP 047 00 for samples

F-1 and D-3, EAWP 047 00 for sample I-1, AAWP 047 00 for sample I-3, DAWP 047 00 for sample S-2, HAWP 047 00 for sample C-2, PHWP 047 00 for samples C-5 and B-3, GSWP 047 00 for samples J-1 and J-3, and VCWP 047 00 for sample E-4.

DMSO solutions of Polytran samples were allowed to stand at 25 - 50°C for 16 - 52 h, and complete dissolution was checked by measuring the viscosity as a function of time. These solutions and the solvent were centrifuged at about 4×10^4 gravities for 2 h.

For both aqueous and DMSO solutions, a central portion of the supernatant of the liquid in the centrifuge tube was sucked into a pipet and transferred into a light scattering cell. The cell and pipet had been rinsed with refluxing acetone vapor for 8 - 12 h.

f. Specific Refractive Index Increment

Refractive indices of Polytran scleroglucan in 0.01 N NaOH and DMSO at 25°C were measured by the method described in Chapter II. The measured Δn in the two solvents are plotted against c in Figure V-2. The indicated straight lines yield 0.145 and 0.142 $cm^3 g^{-1}$ for $\partial n/\partial c$ in 0.01 N NaOH at 436 and 546 nm, respectively,

and 0.063 $cm^3 g^{-1}$ for $\partial n/\partial c$ in DMSO at both 436 and 546 nm.

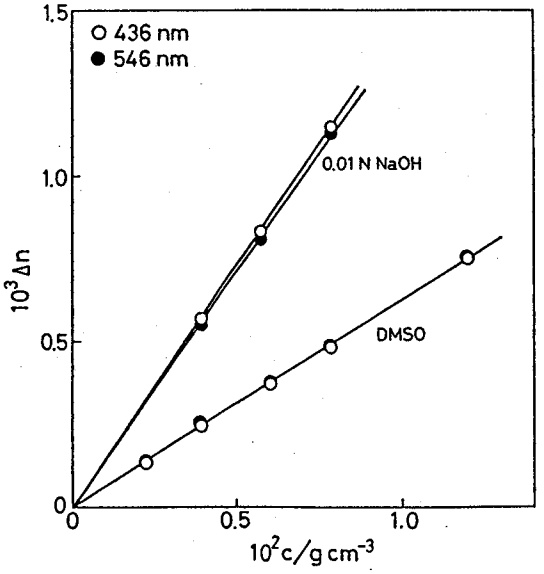

Figure V-2. Excess refractive indices Δn plotted against c for Polytran scleroglucan in 0.01 N aqueous NaOH and DMSO at 25°C.

V-2-5. Ultracentrifugation

Samples B-3 and E-4 in water at 25°C were investigated by sedimentation equilibrium. A Kel-F 12 mm double-sector cell was used. The liquid column was ajusted to 1.4 - 1.6 mm, and the rotor speed was chosen as 5200 and 7200 rpm for samples B-3 and E-4,

respectively. The partial specific volume of Polytran samples in water at 25°C was determined to be 0.620 $cm^3 g^{-1}$.

Sedimentation coefficients of samples C-2, C-5, B-3, J-1, J-3, and E-4 in 0.01 N NaOH at 25°C were determined by the peak method (see Chapter II).

V-2-6. Viscometry

Zero shear-rate viscosities of samples N-2, H-1, F-1, D-3, I-1, I-3, and S-2 in 0.01 N NaOH at 25°C were determined using four-bulb capillary viscometers.[13] For other samples in 0.01 N NaOH and all samples in DMSO at 25°C, conventional capillary viscometers of the Ubbelohde type were used.

The composition dependence of $[\eta]$ in water-DMSO mixtures at 25°C was determined for three samples S-2, C-3, and B-4, and compared with that determined for schizophyllan in Chapter II.

V-3. Results

V-3-1. Chemical Structure

Figure V-3 shows the gas chromatograms of the

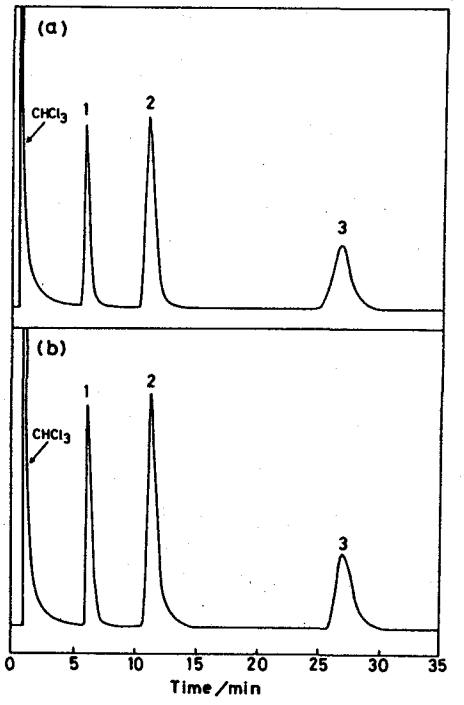

Figure V-3. Gas chromatograms of the acetylated products obtained from methylation experiments. (a), sample N-2; (b), sample S-2.

acetylated products obtained from the methylation experiment. Each product of samples N-2 and S-2 has three peaks 1, 2, and 3; the peak at the shortest retention time corresponds to the solvent chloroform. From the retention times,[14] peaks 1, 2, and 3 were identified as 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol (A), 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-

glucitol (B), and 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-D-glucitol (C), respectively. The molar ratios of products A, B, and C evaluated from the area under the curves are 1.00 : 1.99 : 0.98 for sample N-2 and 1.00 : 2.00 : 1.01 for sample S-2. These ratios indicate that the repeating units of the two samples consist of three 1,3-linked D-glucose residues and one 1,6-linked D-glucose residue.

The results from the periodate oxidation experiment, displayed in Figure V-4, show that each of the two

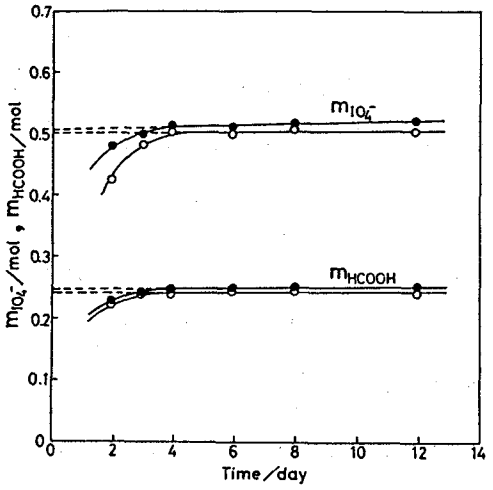

Figure V-4. Amount of periodate $m_{IO_4^-}$ (moles per glucose residue) consumed and that of formic acid m_{HCOOH} produced by periodate oxidation of Polytran samples N-2 (○) and S-2 (●).

samples N-2 and S-2 consumes 0.5 mol of periodate per glucose residue and produces 0.25 mol of formic acid. These values confirm the finding from the methylation experiment that one in every four glucose residues is a 1,6-linked glucopyranose.

Though not shown here, the paper chromatograms of the enzymatic hydrolysates from the Smith-degraded glucans revealed that only glucose was produced by exo-β-1,3-D-glucanase. The implication of this, along with the result from the methylation or oxidation experiment, is that since neither gentiobiose ($G_1\overset{\beta}{-}_6G$) nor isomaltose ($G_1\overset{\alpha}{-}_6G$) could be detected, our scleroglucan consists of repeating units as

$$-\,{}_1G_3-\,{}_1\overset{\overset{G_1}{|}}{G}{}^6_3-\,{}_1G_3-$$

and that all the 1,3-linkages must be of the β-type. Here, G denotes a D-glucose residue and the figures attached to G indicate the modes of linkage.

Figure V-5 shows the paper chromatograms of the enzymatic hydrolysates obtained from samples N-2 and S-2 with exo-β-1,3-D-glucanase. Here, the standard

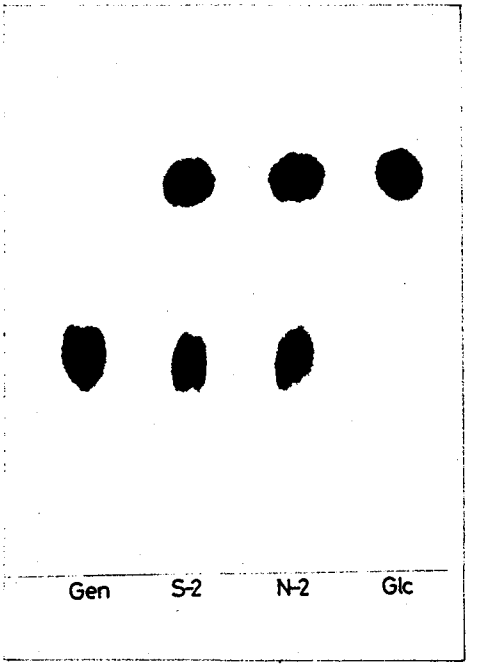

Figure V-5. Paper chromatograms of the enzymatic hydrolysates obtained from Polytran samples N-2 and S-2 with exo-β-1,3-D-glucanase, and those of glucose (Glc) and gentiobiose (Gen) as standard sugars.

spots refer to glucose (Glc) and gentiobiose (Gen). It can be seen that each hydrolysate consists only of gentiobiose and glucose. The molar ratio of glucose to gentiobiose was 1.96 for sample S-2 and 2.08 for sample N-2 when estimated by the phenol-sulfuric acid method of Dubois et al.[15] This result confirms the

repeating unit shown above, and moreover substantiates that the 1,6-linkage in the repeating unit is of the β-type.

Figure V-6 shows the IR spectrum for sample S-2. The absorption band appearing at 890 cm^{-1} is characteristic of the β-glucosidic linkage[16] and consistent with the above finding that Polytran scleroglucan is a β-linked glucan. From the results of the present chemical analysis, we can conclude that the commercially available scleroglucan and its sonicated products consist essentially of the same

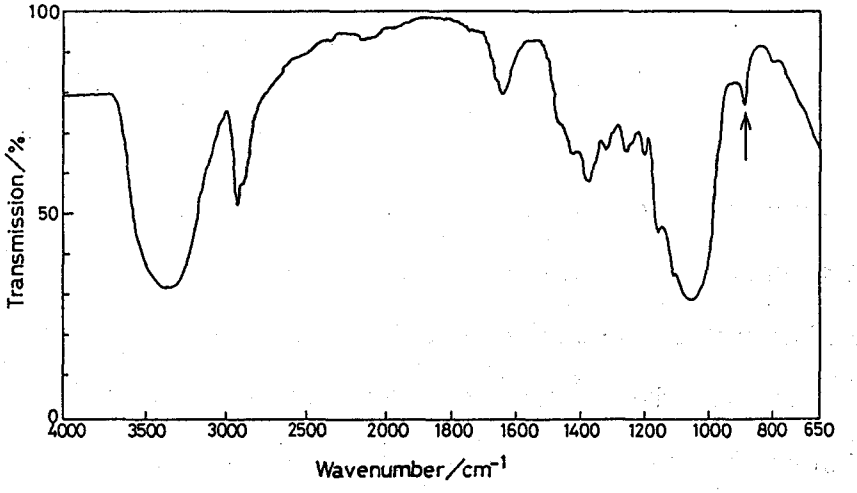

Figure V-6. IR spectrum for Polytran sample S-2.

repeating units as those of schizophyllan. Very recently, Rinaudo and Vincendon[17] arrived at the same conclusion from ^{13}C-NMR measurements.

From the chemical structure so established for our scleroglucan, we further conclude that the Smith-degraded glucan prepared from sample N-2 or S-2 is a curdlan-type β-1,3-D-glucan with no side chain. We determined its [η] in a 1 : 1 mixture of water and tris (ethylene diamine) cadomium dihydroxide (cadoxen) to estimate the viscosity-average molecular weight M_v on the basis of the [η]-M_w relation established by Hirano et al.[18] for curdlan in this mixed solvent. The M_v value obtained was about 70 % of the M_w of the original scleroglucan sample N-2 or S-2 in DMSO (see Table V-1). This percentage is close to 75 % which can be expected if Polytran scleroglucan contains no β-1,6-linkage in the main chain, and supports the conclusion that the main chains of our scleroglucan samples contain only β-1,3-D-glucosidic linkages. Tabata et al.[19] have used this viscosity method to confirm that the same is true for schizophyllan.

V-3-2. Molecular Weight

Figure V-7 illustrates the concentration dependence of Kc/R_0 for samples I-3, C-5, and J-3 in 0.01 N NaOH and DMSO. The values of M_w and A_2 for all the Polytran samples in the two solvents are summarized in Table V-1, along with those of M_w and M_z/M_w in pure water determined by sedimentation equilibrium. The M_z/M_w values for samples B-3 and E-4 indicate that

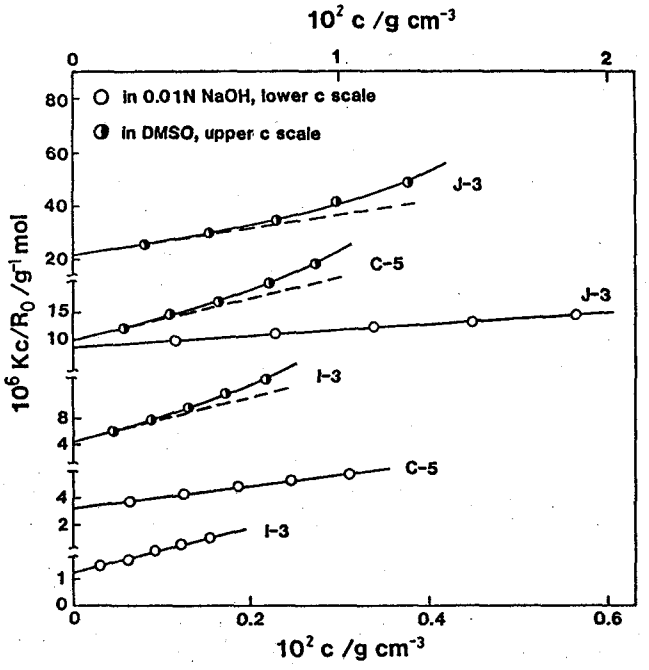

Figure V-7. Plots of Kc/R_0 vs. c for Polytran samples in 0.01 N NaOH (the lower c scale) and DMSO (the upper c scale) at 25°C.

Table V-1. Results from light scattering measurements on Polytran samples in 0.01 N NaOH and DMSO at 25°C

	In 0.01 N NaOH				In DMSO			M_w(in 0.01 N NaOH)
Sample	$10^{-4}M_w$	10^4A_2[a]	$\langle S^2\rangle^{\frac{1}{2}}$/nm	M_z/M_w	$10^{-4}M_w$	10^4A_2[a]	$\langle S^2\rangle^{\frac{1}{2}}$/nm	M_w(in DMSO)
N-2	540	0.6	276	—	143	0.5	57.5	3.8
H-1	370	2.8	264	—	51.8	3.9	46.0	7.1
	163[b]	2.4[b]	151[b]	—	—	—	—	—
F-1	239	5.4	198	—	37.2	5.3	33.1	6.4
	129[b]	3.2[b]	134[b]	—	—	—	—	—
D-3	141	4.4	152	—	29.5	4.3	27.1	4.8
I-1	110	1.7	127	—	25.8	4.9	26.4	4.3
I-3	81.1	4.1	92.7	—	22.5	4.7	23.2	3.6
S-2	60.0	1.8	71.7	—	21.3	0.7	22.6	2.8
C-2	37.6	5.7	51.4	—	12.8	6.8	17.8	2.9
C-5	31.0	4.3	42.1	—	11.0	6.3	16.3	2.8
B-3	22.0	6.2	31.1	—	7.89	6.5	13.8	2.8
	21.2[c]	1.5[c]	—	1.12[c]	—	—	—	—
J-1	16.7	8.0	23.3	—	6.57	5.5	12.6	2.5
J-3	12.0	5.8	17.4	—	4.68	8.3	—	2.6
E-4	9.62	7.8	13.4	—	3.56	6.7	—	2.7
	9.01[c]	3.2[c]	—	1.19[c]	—	—	—	—

[a] In units of $cm^3 mol\ g^{-2}$.

[b] In 0.05 N NaOH.

[c] Sedimentation equilibrium in water.

these two samples are narrow in molecular weight dispersion.

The values of M_w(in 0.01 N NaOH)/M_w(in DMSO) presented in the last column of Table V-1 indicate that for M_w(in DMSO) below 2×10^5, the predominant species of Polytran scleroglucan in 0.01 N aqueous NaOH is a trimer, as is the case for schizophyllan in pure water or 0.01 N aqueous NaOH. However, for M_w(in DMSO) above 2×10^5, the molecular weight ratio increases with increasing M_w(in DMSO), indicating that the major species of the glucan in 0.01 N aqueous NaOH shifts to an aggregate higher than a trimer and the number of chains in the aggregate increases with increasing M_w(in DMSO). This differs from what was found for schizophyllan in Chapter II; Kashiwagi et al.[2] also showed that M_w(in 0.01 N NaOH)/M_w(in DMSO) for schizophyllan exhibited no significant deviation from 3 throughout the entire range of M_w(in DMSO) treated, i. e., 3.7×10^4 - 1.6×10^6.

V-3-3. Radius of Gyration

Figure V-8 depicts P(θ) for Polytran samples in 0.01 N aqueous NaOH. The values of $\langle S^2 \rangle^{1/2}$ evaluated

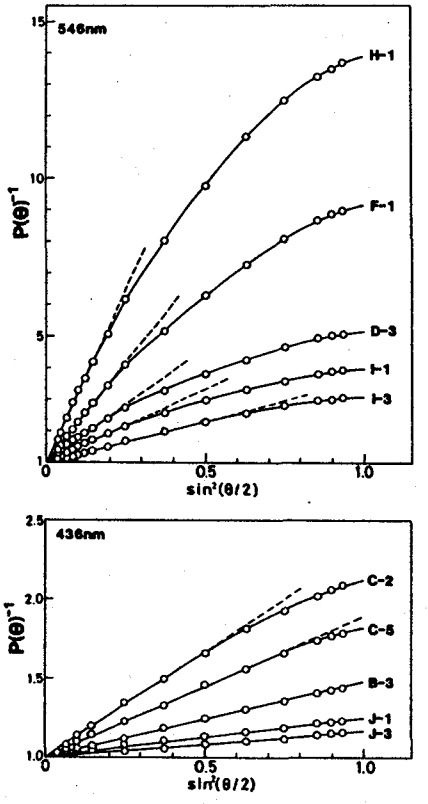

Figure V-8. Particle scattering functions for Polytran samples in 0.01 N NaOH at 25°C.

from the slopes of the indicated dashed lines are presented in Table V-1, along with those in DMSO.

These $\langle S^2 \rangle^{1/2}$ values in 0.01 N aqueous NaOH and DMSO are compared in Figure V-9 with the recent data (the solid lines) of Kashiwagi et al.[2] for schizophyllan in the same solvents. All the data points for Polytran scleroglucan in 0.01 N aqueous NaOH fall near the solid

line for schizophyllan in the same solvent. The slope of this line equals 1.0 for M_w(in 0.01 N NaOH) below 3×10^5 and decreases gradually with increasing M_w(in 0.01 N NaOH). Thus, the scleroglucan trimer [M_w(in 0.01 N NaOH) $< 8 \times 10^5$] is almost rigid-rodlike below and semiflexible above M_w(in 0.01 N NaOH) $\sim 3 \times 10^5$. However, the agreement of $\langle S^2 \rangle^{1/2}$ for both Polytran scleroglucan and schizophyllan in 0.01 N aqueous NaOH

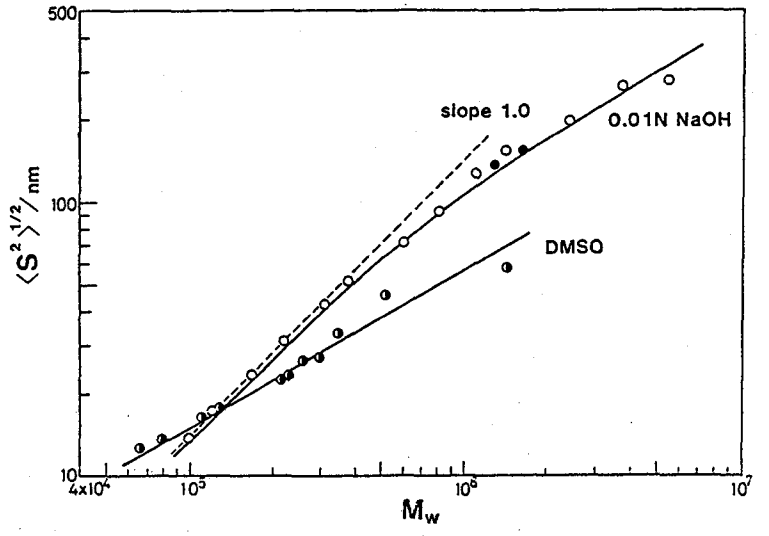

Figure V-9. Comparison of $\langle S^2 \rangle^{1/2}$ data for Polytran scleroglucan in 0.01 N NaOH (○) and DMSO (◑) with those (the solid lines) for schizophyllan[2] in the same solvents. The filled circles represent the data for Polytran samples H-1 and F-1 in 0.05 N NaOH.

throughout the entire range of M_w(in 0.01 N NaOH) must be considered accidental, since, as mentioned above, the predominant species of these glucans in 0.01 N aqueous NaOH are different aggregates when M_w(in DMSO) is higher than about 2×10^5.

In Figure V-9, except for the two highest-molecular weight samples, $\langle S^2 \rangle^{1/2}$ of Polytran scleroglucan in DMSO falls on the straight line of slope 0.58 which fits the schizophyllan data in the same solvent. This indicates that Polytran scleroglucan disperses in DMSO as random coils perturbed by excluded-volume effect as much as schizophyllan random coils in the same solvent.

V-3-4. Intrinsic Viscosity and Sedimentation Coefficient

Numerical data for $[\eta]$ and k' in 0.01 N aqueous NaOH and DMSO are summarized in Table V-2. In Figure V-10, these $[\eta]$ data are compared with those (the solid lines) for schizophyllan[2] in the same solvents. The data points for M_w below 3×10^5 in 0.01 N aqueous NaOH can be fitted by a straight line of slope 1.7 (the dashed line), which almost merges with the solid line for this solvent and is consistent with the

Table V-2. Results from viscosity and sedimentation velocity measurements on Polytran samples in 0.01 N NaOH and DMSO at 25°C

	In 0.01 N NaOH				In DMSO	
Sample	$10^{-2}[\eta]/cm^3g^{-1}$	k'	$10^{13}s_0/s$	$10^{-2}k_s/cm^3g^{-1}$	$10^{-2}[\eta]/cm^3g^{-1}$	k'
N-2	65.8	0.45	—	—	2.43	0.34
H-1	43.7	0.45	—	—	2.17	0.32
	25.3[a]	0.44[a]	—	—	—	—
F-1	26.1	0.43	—	—	1.80	0.34
	17.0[a]	0.44[a]	—	—	—	—
D-3	14.2	0.42	—	—	1.39	0.34
I-1	10.5	0.43	—	—	1.26	0.36
I-3	6.59	0.41	—	—	1.09	0.34
S-2	4.59	0.43	—	—	0.932	0.37
C-2	2.65	0.41	7.42	75	0.781	0.35
C-3	2.61	0.41	—	—	0.770	0.36
C-5	2.03	0.42	7.17	64	0.740	0.33
B-3	1.34	0.42	6.80	62	0.562	0.38
B-4	1.30	0.42	—	—	0.558	0.36
J-1	0.809	0.42	6.29	53	0.462	0.35
J-3	0.497	0.43	5.73	42	0.376	0.40
E-4	0.345	0.44	5.34	34	0.305	0.42

[a] In 0.05 N NaOH.

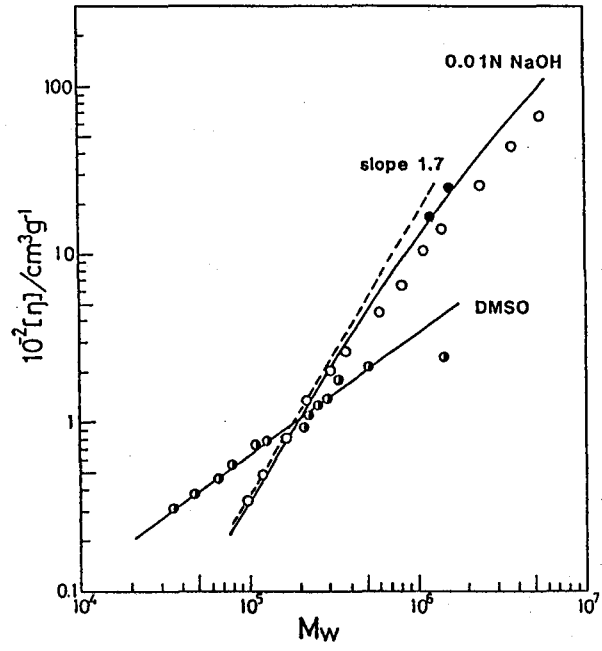

Figure V-10. Comparison of [η] data for Polytran scleroglucan in 0.01 N NaOH (○) and DMSO (◑) with those (the solid lines) for schizophyllan[2] in the same solvents. The filled circles represent the data for Polytran samples H-1 and F-1 in 0.05 N NaOH.

finding from $\langle S^2 \rangle$ that scleroglucan trimers with M_w (in 0.01 N NaOH) lower than 3×10^5 are rigid-rodlike. On the other hand, except for the highest-molecular weight sample, the DMSO data fall on the schizophyllan line of slope 0.69, giving evidence for similarity between scleroglucan and schizophyllan random coils in

this solvent.

Figure V-11 shows the concentration dependence of s^{-1} for Polytran samples in 0.01 N aqueous NaOH. The values of s_0 and k_s determined from the indicated straight lines are presented in the fourth and fifth columns of Table V-2. Figure V-12 shows that these s_0 vary linearly with log M_w. This linear relation is additional evidence for the rigid rod-like shape of the scleroglucan trimer.

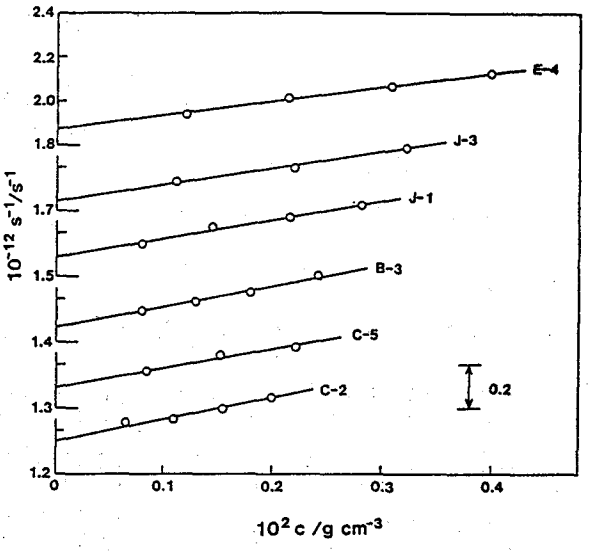

Figure V-11. Concentration dependence of s^{-1} for Polytran samples in 0.01 N NaOH at 25°C.

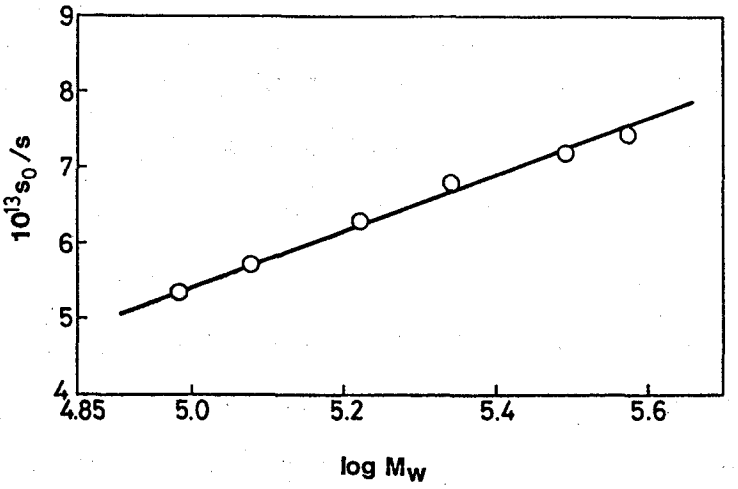

Figure V-12. Plot of s_0 vs. log M_w for Polytran scleroglucan in 0.01 N NaOH.

V-3-5. Viscosity Behavior in Water-DMSO Mixtures

Figure V-13 shows the composition dependence of [η] of samples S-2, C-3, and B-4 in water-DMSO mixtures at 25°C. The [η] values in water are approximately equal to those in 0.01 N NaOH (see Table V-2). Hence, the conformations of Polytran scleroglucan in pure water and 0.01 N NaOH are essentially identical (see also the M_w values in pure water and 0.01 N NaOH given in Table V-1). As the composition of DMSO increases, [η] for each sample gradually increases, passes through a broad maximum, and decreases very sharply at a DMSO

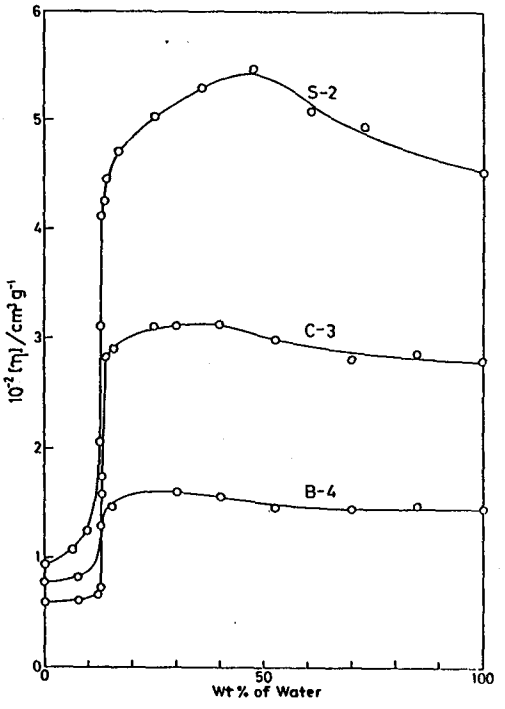

Figure V-13. Composition dependence of $[\eta]$ for indicated Polytran samples in water-DMSO mixtures at 25°C.

composition of about 87 %. At present, we do not know what is responsible for the appearance of the maximum. What is more significant is the abrupt drop of $[\eta]$ taking place at exactly the same DMSO composition as that at which schizophyllan triple helices dissociate almost discontinuously to single chains (see Figure III-2 in Chapter III).

V-4. Discussion

V-4-1. Data Analysis

Our dimensional and hydrodynamic data along with those of the molecular weight ratio M_w(in 0.01 N NaOH)/ M_w(in DMSO) have shown that Polytran scleroglucan dissolves in 0.01 N aqueous NaOH as a rigid trimer, provided M_w(in DMSO) is lower than 1×10^5. The well-known expression[20] for $\langle S^2 \rangle$ of a rigid rod is

$$\langle S^2 \rangle^{1/2} = M/(\sqrt{12}\, M_L) \qquad (5\text{-}10)$$

If our $\langle S^2 \rangle$ data for M_w(in DMSO) below 1×10^5 in 0.01 N aqueous NaOH are substituted into this equation, M_L is found to be 2050 ± 50 nm^{-1}.

Figure V-14 shows the plot of $M_w^2/[\eta]$ vs. ln M_w constructed from our data for four lowest-molecular weight samples in 0.01 N NaOH. If a straight line is drawn as indicated and the values of its slope and intercept at ln $M_w = 0$ are substituted into eq 2-11, M_L and d are found to be 2200 ± 60 nm^{-1} and 2.6 ± 0.4 nm, respectively. Further, when compared with eq 2-12, the linear relation between s_0 and log M_w in Figure V-12

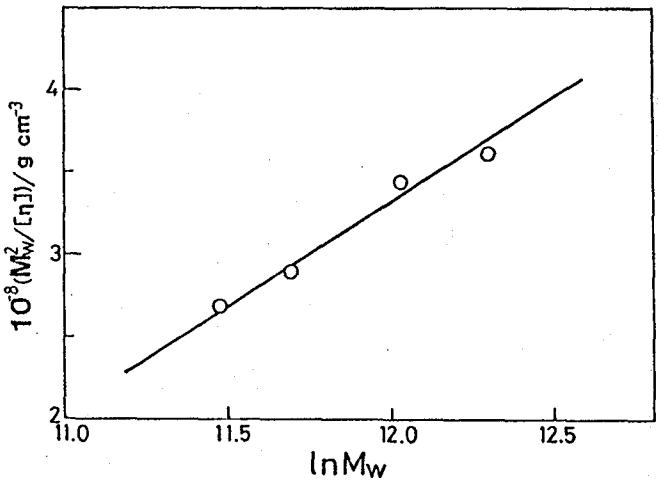

Figure V-14. Plot of $M_w^2/[\eta]$ vs. ln M_w for Polytran scleroglucan in 0.01 N NaOH.

yields 2140 ± 70 nm^{-1} for M_L and 2.5 ± 0.4 nm for d.

V-4-2. Triple Helical Structure

Since the repeating unit of Polytran scleroglucan contains three main chain glucose residues, the length h per main chain residue of the scleroglucan trimer along the rod axis is related to M_L by eq 2-13. The values of h calculated from our M_L and those of d estimated above are summarized in Table V-3, along with the pitches and diameters for the schizophyllan triple helix in water (see Chapter II) and 0.01 N NaOH and the

Table V-3. Pitches and diameters of the triple helices of Polytran scleroglucan and schizophyllan

Polysaccharide	Solvent	Pitch[a]/nm or h/nm	d/nm	Method
Polytran scleroglucan	0.01 N NaOH	0.32 ± 0.01		light scattering
		0.29 ± 0.01	2.6 ± 0.4	viscosity
		0.30 ± 0.01	2.5 ± 0.4	sedimentation
	crystalline state	0.30[a]		X-ray[22]
Schizophyllan	water	0.30 ± 0.02[a]	2.6 ± 0.4	viscosity & sedimentation[b]
	0.01 N NaOH	0.30 ± 0.01[a]	2.2 ± 0.6	viscosity & light scattering[2]
	———	0.30[a]	2.5 - 3.0	molecular model[b]
	crystalline state	0.30[a]		X-ray[23]

[a] Per residue.

[b] See Chapter II.

model triple helix of schizophyllan (see Chapter II). The h values for the scleroglucan trimer estimated by different methods agree with one another and also with the schizophyllan helix pitches. The d values are also in substantial agreement with those for schizophyllan and its model helix. Thus, the scleroglucan trimer should have a triple helical structure very similar to that of schizophyllan. This conclusion is consistent with our chemical analysis data showing that the repeating unit of Polytran scleroglucan is identical with that of schizophyllan.

Very recently, Marchessault et al.[21,22] concluded from x-ray and conformational studies that Polytran scleroglucan in the crystalline state has a triple helical structure with a pitch (per residue) of 0.30 nm. This value can be favorably compared with the values from our solution study, and hence it may be concluded that the triple helical conformation of Polytran scleroglucan in the crystalline state is essentially the same as that in dilute aqueous NaOH.

V-4-3. Aggregates of Triple Helices

As we have mentioned, our scleroglucan samples

with M_w(in DMSO) higher than 2 x 10^5 in 0.01 N aqueous NaOH self associate to aggregates higher than a trimer. In the following, we present some data which convince us that these higher aggregates are built up of two or more trimers.

According to Kashiwagi et al.,[2] the addition of NaOH enhances the solubility of the schizophyllan triple helix in water, but it causes the helix to dissociate to single chains and also gradually degrade when the NaOH concentration x exceeds 0.1 N. We checked the former on Polytran scleroglucan by measuring the viscosity of higher-molecular weight samples H-1 and F-1 and lower-molecular weight samples B-3 and J-1 in aqueous NaOH of different x.

The results are illustrated in Figure V-15. It can be seen that $[\eta]$ for samples B-3 and J-1 are constant up to x $\sim$ 0.05 N, decrease sharply at x $\sim$ 0.1 N, and level off at constant values above x $\sim$ 0.2 N. The abrupt change in $[\eta]$ at x $\sim$ 0.1 N may be due primarily to the dissociation of the triple helix to single chains, since $[\eta]$ for x above 0.2 N is comparable to $[\eta]$ of the respective samples in DMSO. The curves for samples H-1 and F-1 comprise two distinct steps, each

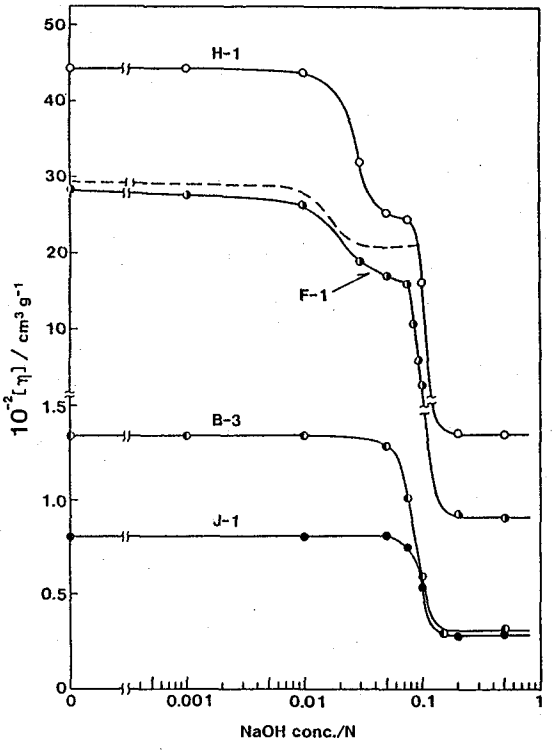

Figure V-15. Dependence of [η] on the concentration of NaOH for Polytran samples in aqueous NaOH at 25°C. The dashed line indicates the data of Bluhm et al.[22]

showing a shoulder around x = 0.05 N. Probably, in the decline to the shoulder the higher aggregates are dissociated to trimers, and in the subsequent sharp decline, the trimers are broken to single chains.

To check this inference, light scattering and viscosity measurements were made on samples H-1 and F-1

in 0.05 N aqueous NaOH. The specific refractive index increment in this solvent at either 436 or 546 nm wavelength was not different from that in 0.01 N aqueous NaOH. The data obtained are given in Tables V-1 and V-2 and illustrated in Figures V-9 and V-10. The filled circles in these figures lie close to the solid lines for schizophyllan in 0.01 N aqueous NaOH and also give M_w values approximately 3 times as large as those in DMSO. This demonstrates dissociation of the aggregates of samples H-1 and F-1 in 0.05 N NaOH to trimers having the same structure as the schizophyllan triple helix.

Recently, Bluhm et al.[22] investigated the pH dependence of the reduced viscosity η_{sp}/c ($c = 4.9 \times 10^{-4}$ g cm^{-3}) of a Polytran sample in 8×10^{-2} M sodium chloride containing NaOH (for pH > 7) or hydrochloric acid (for pH < 7). Their experimental data are shown by a dashed line in Figure V-15 after pH has been converted to x assuming the relation log x = pH - 14. Bluhm et al. remarked that the values of η_{sp}/c for x above 0.03 N are comparable to our $[\eta]$ for schizophyllan in DMSO, and attributed a fairly sharp decrease in η_{sp}/c at $x \sim 0.015$ N to the dissociation of triple helices to random coils. However, their remark on η_{sp}/c

for x above 0.03 N is incorrect, since such η_{sp}/c values far exceed the highest of $[\eta]$ ($4.65 \times 10^2 cm^3 g^{-1}$) obtained for schizophyllan in DMSO (see Table II-2 in Chapter II). More reasonably, the sharp decrease in η_{sp}/c at $x \sim 0.015$ N may be explained as due to the dessociation of higher aggregates to trimers.

V-5. Concluding Remarks

The study in this chapter has shown that the trimer of Polytran scleroglucan in dilute aqueous NaOH or pure water and its monomer in DMSO assume essentially the same conformations as those of schizophyllan in these solvents, and that when dissolved in water-DMSO mixtures, the trimers of these two glucans dissociate into monomers at the same water composition. These findings are compatible with the results from our chemical analysis and Rinaudo-Vincendon's ^{13}C-NMR measurements,[17] which concluded that the two polysaccharides have the same chemical structure. However, these polymers in 0.01 N aqueous NaOH differed in solubility when M_w(in DMSO) was higher than 2×10^5. In this region of M_w, triple helices of schizophyllan remained intact, while those of Polytran scleroglucan self associated to higher

aggregates. This difference is striking, but the reason is not clear to us. Native schizophyllan produced by Schizophyllum commune in a culture medium separates spontaneously from the mycelium and migrates freely into the liquid phase.[24] On the other hand, Polytran scleroglucan produced by Sclerotium rolfsii adheres to the mycelium as a gel-like aggregate and cannot be dispersed in the liquid phase unless the culture medium is heated and homogenized.[1] Such a difference in the native states of these two glucans may bear some relation to the difference in their solubility in 0.01 N NaOH.

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24. ref. 6 in Chapter I.

CHAPTER VI

CHOLESTERIC MESOPHASE IN AQUEOUS SOLUTIONS OF POLYTRAN SCLEROGLUCAN

VI-1. Introduction

Recently, Van et al.[1] found that schizophyllan in water forms a cholesteric mesophase at a polymer concentration above about 10 %. In the preceding chapter, we showed that Polytran scleroglucan is very similar to schizophyllan in regard to chemical structure and triple-helical structure in pure water or dilute aqueous sodium hydroxide. These similarities between the two glucans suggest that aqueous scleroglucan should form a cholesteric mesophase when the concentration is above a certain value.

We investigated aqueous solutions of two samples of Polytran scleroglucan over a concentration range from zero to about 35 % by polarizing microscopy and optical rotatory dispersion (ORD). The results obtained are presented in this chapter.

VI-2. Experimental

Two Polytran samples were prepared by sonication and fractionated by the methods described in Chapter II. Two fractions, one from each sample, were chosen and designated as L-2 and P-3. Their M_v in water were 1.93 x 10^5 (L-2) and 1.24 x 10^5 (P-3).

Test solutions were prepared by mixing weighed amounts of a given sample and water in a 5 cm^3 stoppered flask. They were transferred into drum-shaped cells of 1 mm thickness and 1 cm diameter and examined under a Union Mec-3 polarizing microscope. The polymer concentration was expressed in terms of the weight fraction w or the volume fraction ϕ; the latter was calculated from w with the specific volume of Polytran scleroglucan and the density of water.

ORD measurements were made on sample L-2, using a JASCO ORD/UV-5 recording spectropolarimeter. Preliminary experiments were performed on solutions with w above 0.2, using rectangular cells of different thicknesses d (20 - 350 μm) constructed according to Van et al.[2] The results showed that, as in schizophyllan liquid crystals,[2] $[\alpha]_\lambda$ at a fixed λ was independent of d and reproducible within ± 5 % only when d was

smaller than about 100 μm. Thus, rectangular cells of d ∿ 80 μm were used for solutions with w above 0.15. For solutions with w below 0.15, usual ORD cells 0.5 - 10 cm thick were used.

VI-3. Results and Discussion

VI-3-1. Microscopic Observation

Aqueous solutions of sample L-2 at 25°C looked dark throughout the field between crossed polars when w was lower than 0.14. This indicates that the solutions in this concentration region are isotropic. At w slightly higher than 0.14, birefringent phases with alternate bright and dark lines appeared as spherulites dispersed in a continuous isotropic phase a few hours after the solution had been placed in the cell. Figure VI-1 shows a spherulite photographed for the L-2 solution with w = 0.1567 at 25°C.

When w was increased to about 0.2, the birefringent phases spread over the entire region of the solution and showed various colors. Figure VI-2 shows microscopic patterns for the birefringent solution of sample L-2 with w = 0.2089 at 25°C near the wall [panel

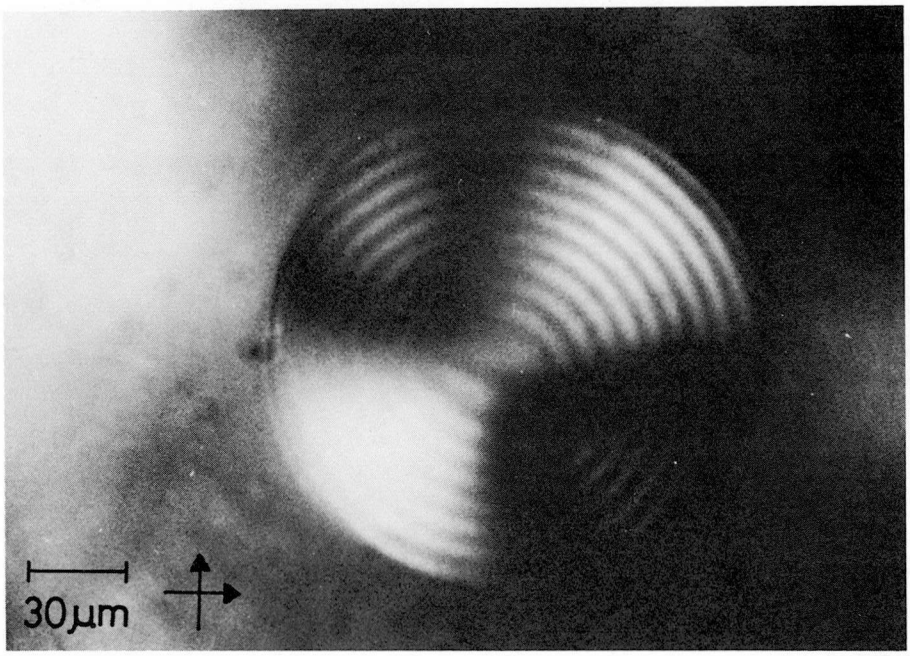

Figure VI-1. Spherulite in the aqueous solution of Polytran sample L-2 with w = 0.1567 at 25°C. The bright region on the top left-hand side shows the reflection of light from another large spherulite in the solution.

(a)] and center [panel (b)] of the cell. It can be seen that the bright and dark lines in panel (a) run parallel to the cell wall, while those in panel (b) run in different directions to form fingerprint patterns. These patterns resemble those observed for the cholesteric liquid crystals of polypeptides[3-5] and polysaccharides.[1,6,7]

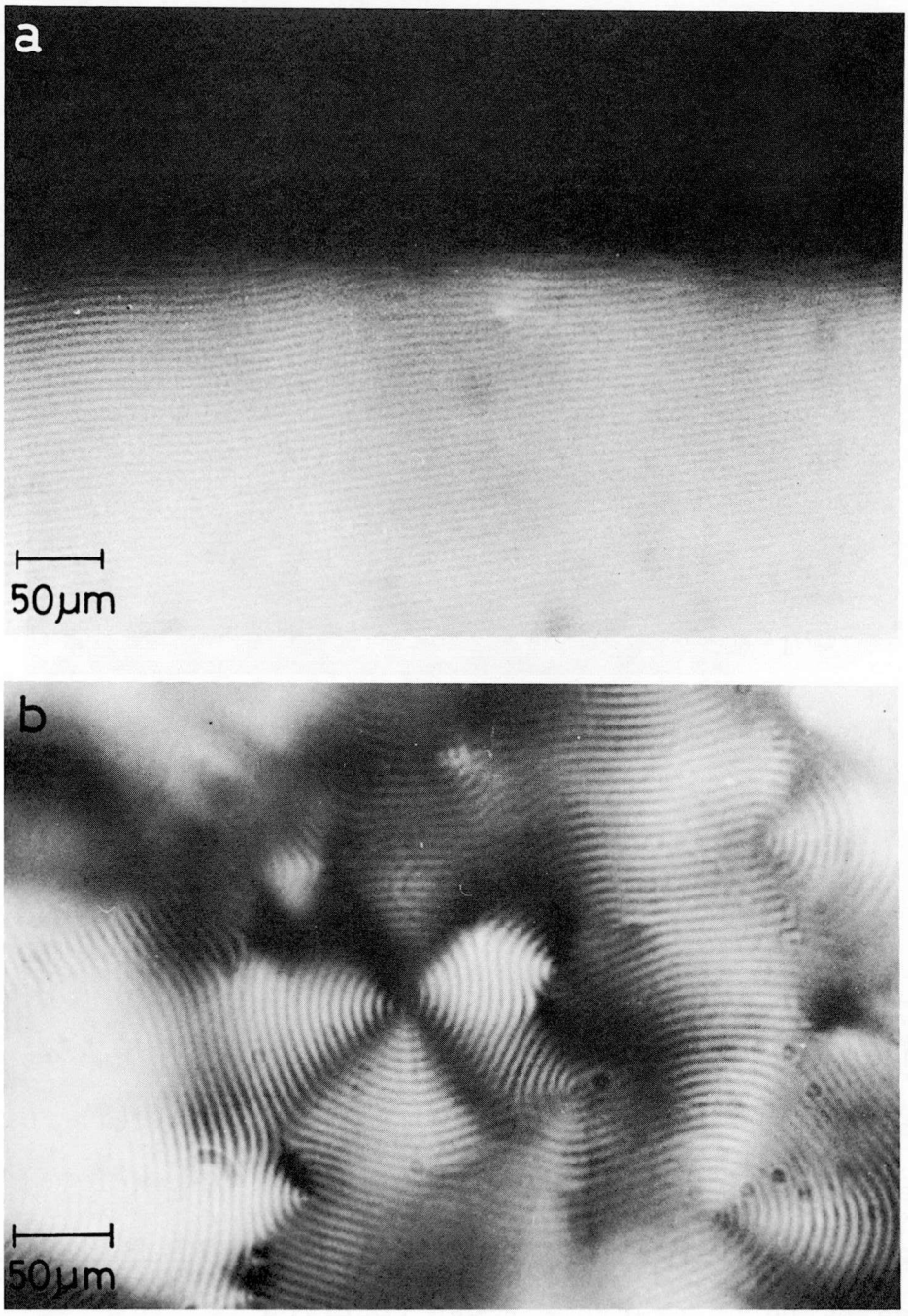

Figure VI-2. Microscopic patterns for the birefringent solution of sample L-2 with w = 0.2089 at 25°C. (a), near the cell wall; (b), near the center of the cell.

Similar observations made at different temperatures between 20 and 25°C for samples L-2 and P-3 yielded results almost independent of temperature.

For a given biphasic or entirely birefringent solution, the distance S between successive parallel lines was constant except near the cell wall. Also, S remained invariant at least for one month.

The measured values of S for the two Polytran samples at 25°C are plotted double-logarithmically against w in Figure VI-3. Here, the half-filled and

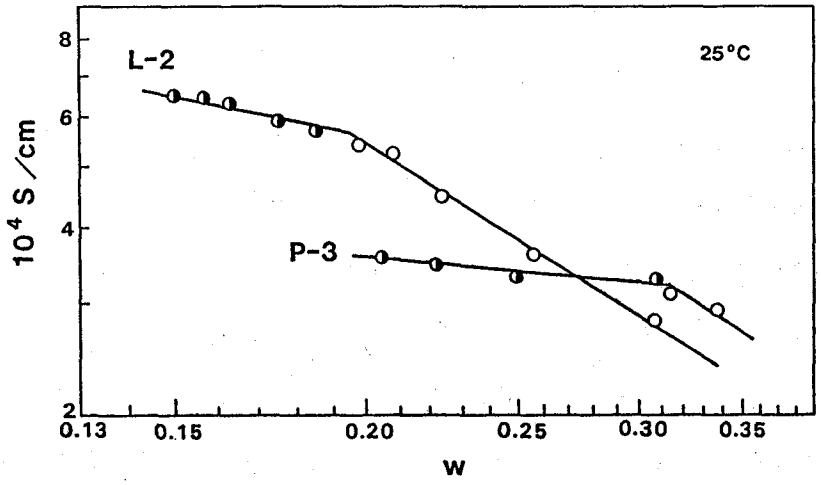

Figure VI-3. Double-logarithmic plots of S vs. w for samples L-2 and P-3 at 25°C. (◑), biphasic solutions; (○), birefringent solutions.

unfilled circles represent the data for biphasic and birefringent solutions, respectively. The data points for sample L-2 can be fitted by a pair of straight lines intersecting at w = 0.194. The branch for $w \geq 0.194$ has a slope of -1.6, which is fairly close to -1.9 reported for schizophyllan.[1] The line for birefringent solutions of sample P-3 has been drawn with the same slope of -1.6. It is interesting to note that when compared at the same w, S for birefringent solutions of the two Polytran samples are about twice as large as those for schizophyllan[8] with comparable molecular weights.

VI-3-2. Optical Rotatory Dispersion

Figure VI-4 displays ORD curves for solutions of sample L-2 with different w at 21.5°C. The curves for $w \geq 0.160$ are very different from those for $w \leq 0.140$. The values of $[\alpha]_{436}$ and $[\alpha]_{589}$ at 436 and 589 nm read from these curves are plotted against w in Figure VI-5. With increasing w, $[\alpha]_{436}$ and $[\alpha]_{589}$ become independent of w following a sharp rise in the region of w between 0.14 and 0.19. Since w = 0.14 almost coincides with the critical concentration (for sample L-2) for the

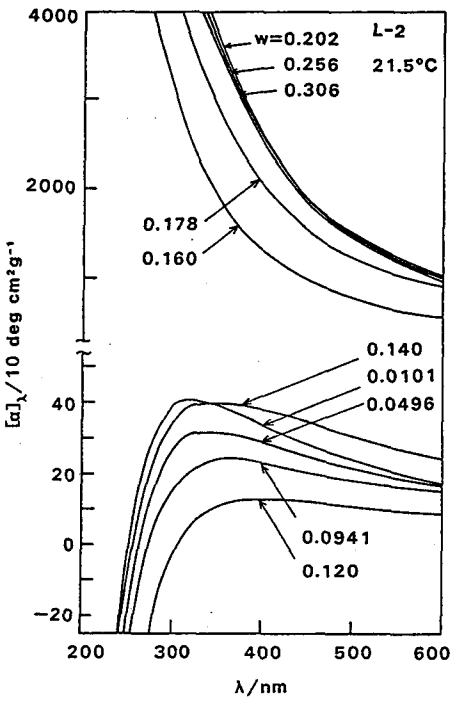

Figure VI-4. ORD curves for aqueous solutions of sample L-2 with different w at 21.5°C.

inception of spherulites in the continuous isotropic phase, the sharp increases in $[\alpha]_{436}$ and $[\alpha]_{589}$ may be explained as due to the formation of birefringent phases. Furthermore, the level-off values of $[\alpha]_{\lambda}$ (about 200 and 100 deg $g^{-1}cm^2$ for 436 and 589 nm, respectively) are 70 - 50 times the infinite dilution values and comparable to those for the cholesteric

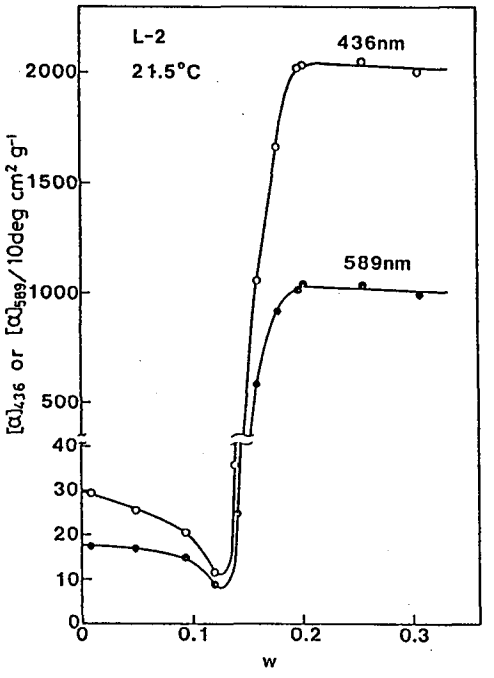

Figure VI-5. Concentration dependence of $[\alpha]_{436}$ and $[\alpha]_{589}$ for sample L-2.

liquid crystal of schizophyllan.[2] From these findings along with the microscopic patterns for Polytran scleroglucan similar to those for cholesteric liquid crystals, it may be concluded that the birefringent phase in aqueous scleroglucan consists of cholesteric liquid crystals.

Figure VI-6 illustrates ORD data for the L-2 solution of w = 0.140 at different temperatures.

It can be seen that $[\alpha]_\lambda$ at a fixed λ remarkably increases with a decrease in temperature from 21.5 to 15°C. When observed under a polarizing microscope, this solution exhibited birefringent phases at 15 and 14°C, but not at 21.5 and 25°C. These findings suggest that aqueous scleroglucan undergoes a thermally induced isotropic liquid-liquid crystal phase transition.

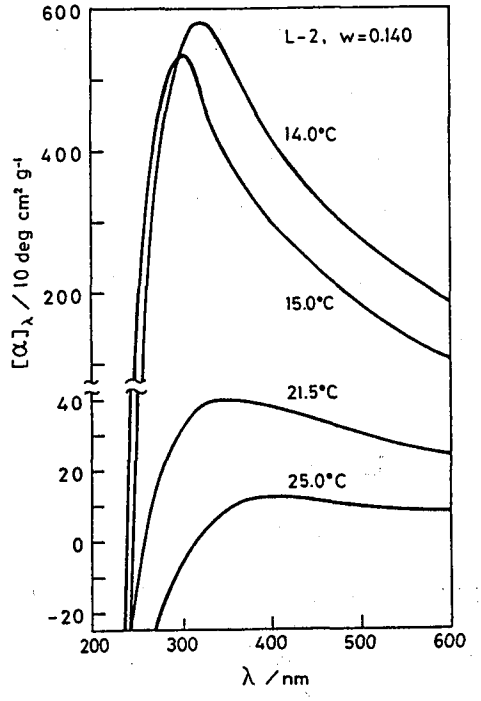

Figure VI-6. ORD curves for the solution of sample L-2 with w = 0.140 at different temperatures.

VI-3-3. Phase Diagram

Our microscopic observations and ORD results indicate that the phase boundary between isotropic and biphasic regions (the A point) and that between biphasic and liquid crystalline regions (the B point) at room temperature are located at $w \sim 0.14$ and 0.19 for sample L-2 and $w \sim 0.19$ and 0.31 for sample P-3. Referring to these w values, we determined temperature-concentration phase diagrams for the two Polytran samples by the methods described below.

a. Microscopic Determination

A solution of sample L-2 with $w \sim 0.14$ and that of sample P-3 with $w \sim 0.19$ were examined between crossed polars, after being equilibrated at different T between 11 and 33°C. The results are illustrated in Figure VI-7, in which the filled circles refer to isotropic solutions at given T and w, and the unfilled circles to biphasic solutions. The line passing between these filled and unfilled circles for each sample describes the A point. The B point concentrations estimated from the break points of the S vs. w curves in Figure VI-3 are also shown in Figure VI-7 by half-filled circles.

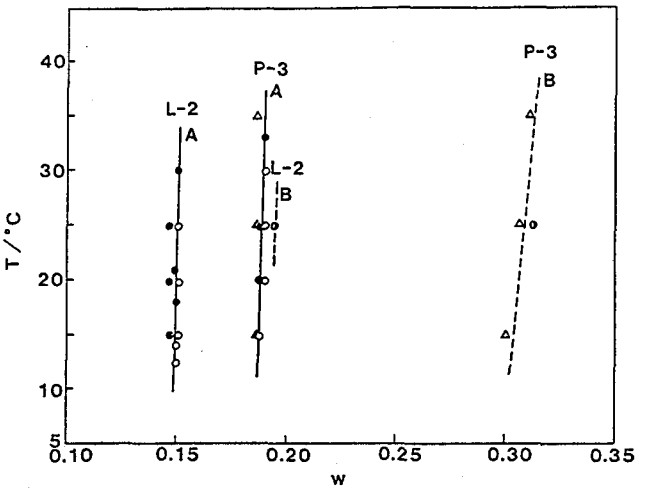

Figure VI-7. Phase diagrams for samples L-2 and P-3. Circles, from microscopic observations; triangles, from phase separation experiments.

b. Analysis of Biphasic Mixtures

When a biphasic solution placed in a calibrated glass tube[8] was spun at a rotor speed of 4000 rpm (about 2000 gravities) for 1 - 2 h in a Hitachi 65P-7 preparative ultracentrifuge, it separated completely to isotropic and liquid crystalline phases. The volume fraction Φ of the isotropic phase and the molecular weight and weight fraction of the polymer in each phase were determined by the method of Itou et al.[8] under the assumption that redistribution of polymer molecules by

centrifugal force was negligible.

Figure VI-8 illustrates the results for sample L-2 at 25°C. Here, the half-filled circles refer to the biphasic mixtures before separation, and the filled and unfilled circles to the separated isotropic and

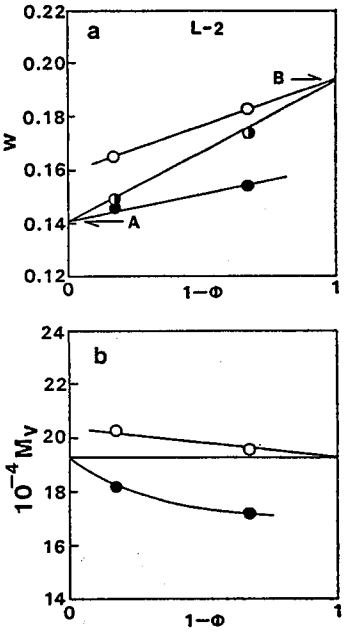

Figure VI-8. Phase separation data for sample L-2 at 25°C. (◑), biphasic mixtures; (●) and (○), separated isotropic and cholesteric solutions, respectively. Arrows A and B indicate A and B point concentrations determined by polarizing microscopy.

cholesteric phases, respectively. It can be seen in panel (a) that extrapolation of w for the biphasic mixtures and separated phases to Φ = 1 and 0 yields phase boundary concentrations very close to the microscopically determined values (arrows A and B). In panel (b), M_v in the liquid crystalline phase are larger than those in the isotropic phase, which indicates that sample L-2 undergoes molecular weight fractionation on phase separation.

The phase boundary w estimated from similar data for sample P-3 are shown in Figure VI-7 by triangles. These w agree with the microscopically determined values. Except for the B point concentrations for sample P-3, all the phase boundary concentrations for Polytran scleroglucan are close to the reported values for schizophyllan with comparable molecular weights.[8]

VI-3-4. Birefringence of the Cholesteric Layer

When cholesteric solutions of sample L-2 in rectangular cells of d $\sim$ 80 μm were examined microscopically between crossed polars, they looked entirely uniform. This implies that, in these cells, all the cholesteric planes were parallel to the cell

surfaces. Thus, our ORD data for w above the B point refer to the geometrical condition that the cholesteric planes are aligned in the direction normal to incident light, and may be analyzed by de Vries' theory[9] for Θ (the optical ratation angle per unit length) of a cholesteric liquid crystal satisfying this condition.

The de Vries theory is written

$$\Theta = (\pi/4\lambda^2)(\Delta n)^2 P \qquad (6\text{-}1)$$

provided that $\Delta nP \ll \lambda \ll P$. Here, P is the cholesteric pitch (= 2S) and Δn is the cholesteric layer birefringence defined by $(n_{\parallel} - n_{\perp})$, with $n_{\parallel}$ and $n_{\perp}$ the refractive indices of a layer in the longitudinal and transverse directions of the director, respectively.

A test of eq 6-1 is shown in Figure VI-9 where Θ from our ORD data on sample L-2 is plotted against λ^{-2}. The curve fitting the plotted points for each w is linear at small λ^{-2} but swings upward for λ^{-2} above 3.5×10^8 cm^{-2}. Equating the initial slope to $\pi P(\Delta n)^2/4$ and using the microscopically determined 2S for P, we evaluated $|\Delta n|$ as a function of w.

The $|\Delta n|$ values so obtained were checked by

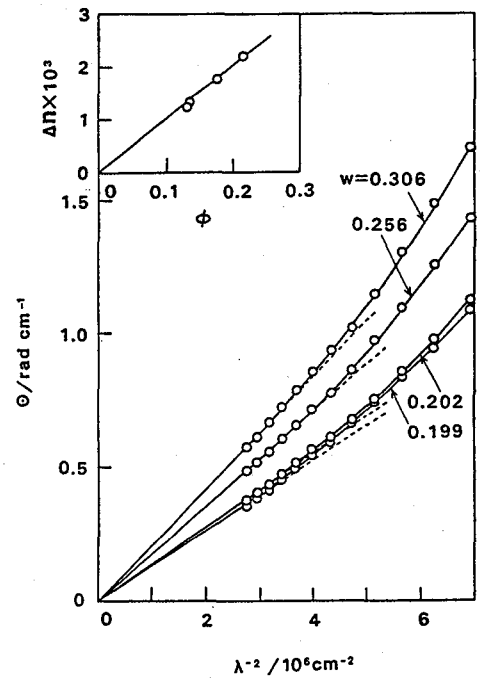

Figure VI-9. Plots of Θ vs. λ^{-2} for sample L-2. The insert shows the dependence of the layer birefringence Δn on polymer volume fraction.

retardation measurements (see ref. 2 for the experimental method). It was found that, though not accurate, the values of Δn from retardation were positive and close to Δn from Θ. This agreement confirms the conclusion of Van et al.[2] that eq 6-1 is valid for longer wavelength.

The insert of Figure VI-9 shows that Δn from Θ varies almost linearly with polymer volume fraction φ.

The indicated straight line yields a value of 1.0×10^{-2} for $|\Delta n|/\phi$ which is close to 1.4×10^{-2} reported for schizophyllan,[2] but much smaller than 4×10^{-2} for xanthan,[7] a β-1,4-D-glucan with ionic side chains. According to Van et al.,[2] Δn is related to the intrinsic birefringence $(n_\alpha - n_\gamma)$ of individual cylindrical macromolecules constituting a cholesteric layer by

$$\Delta n = (n_\alpha - n_\gamma)\phi\zeta(\phi) \qquad (6\text{-}2)$$

where n_α and n_γ are the principal refractive indices in the longitudinal and transverse directions of the cylindrical molecule, respectively, and $\zeta(\phi)$ is a parameter characterizing the degree of molecular ordering in cholesteric solutions. Thus, our $\Delta n/\phi$ value gives $n_\alpha - n_\gamma$ of the scleroglucan triple helix if $\zeta(\phi)$ is unity in the range of ϕ studied. At present, however, no information is available on $\zeta(\phi)$ for cholesteric liquid crystals.

Doi[10] theoretically predicted that $\zeta(\phi)$ for a nematic liquid crystal monotonically increases from 0.5 to unity as ϕ increases from the value at the B point toward unity. When Doi's $\zeta(\phi)$ was applied to our Δn

data, $(n_\alpha - n_\gamma)$ for Polytran scleroglucan decreased from 1.7×10^{-2} to 1.3×10^{-2} with increasing ϕ. If this is taken literally, $\zeta(\phi)$ for Polytran scleroglucan is less dependent on ϕ than Doi's theoretical prediction.

However, too much credit cannot be given to this finding, because Doi's theory is concerned with nematic liquid crystals and de Vries' theory can describe experimental data only in a limited wavelength range. Thus, it is probably safe to conclude that the intrinsic birefringence of the scleroglucan triple helix is about 1.5×10^{-2} and indistinguishable from that of the schizophyllan triple helix.

VI-4. Concluding Remarks

We have found that the cholesteric liquid crystal of aqueous Polytran scleroglucan resembles that of aqueous schizophyllan in thermodynamic and optical properties. This is consistent with the finding in the preceding chapter that these two glucans are chemically indistinguishable and have essentially the same triple helical structures.

However, a distinct difference in S (hence in P) between the two glucans cannot be overlooked; P for

Polytran scleroglucan is almost twice that for schizophyllan when compared at the same w. A cholesteric liquid crystal may be modelled as a pile of thin layers,[11] which are successively twisted by a small angle δ. The two liquid crystals may differ in interlayer distance, in δ, or in both. If the interlayer distance is the same for the two glucans, δ for Polytran scleroglucan should be roughly one half that for schizophyllan. In any case, the two liquid crystals must be different in interlayer interaction. This may be related to the difference in solubility between the two glucans as found in Chapter V.

As can be seen in Figure VI-9, our ORD data follow de Vries' prediction only in a limited range of λ^{-2}. We found that, within the wavelength range examined, the ORD curves for our cholesteric solutions can be fitted accurately by the Drude equation, as was the case for the liquid crystals of schizophyllan[2] and poly(γ-benzyl L-glutamate).[12] Further theoretical study is needed for clarifying this characteristic ORD behavior of polymer liquid crystals.

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CHAPTER VII

SUMMARY AND CONCLUSIONS

This thesis has dealt with the solution properties of two β-1,3-D-glucans, schizophyllan and Polytran scleroglucan, in water or 0.01 N aqueous NaOH and DMSO. The main results and conclusions are summarized below.

Chapter II. Triple Helix of Schizophyllan in Dilute Aqueous Solution

Light scattering, viscosity, and sedimentation measurements on a series of native and sonicated samples of schizophyllan led to the following results. (i) Both weight-average and z-average molecular weights ranging from 10^5 to 6×10^6 in water are about three times as large as those in DMSO. (ii) The viscosity exponent in the Houwink-Mark-Sakurada equation[1] in water is close to 1.7 for M_w(in water) below 5×10^5, and gradually decreases with increasing M_w, while that in DMSO is 0.68 throughout the entire range of M_w studied. (iii) The

limiting sedimentation coefficient in water varies linearly with log M_w for M_w(in water) below 10^6.

These results were combined to conclude that schizophyllan dissolves as rodlike trimers in water and as single randomly coiled chains in DMSO. The molecular model constructed for the schizophyllan trimer suggested that three chains in the trimer are in a triple-stranded helix with a pitch (per main chain residue) of 0.30 nm and a diameter of 2.5 - 3.0 nm.

Analysis of $[\eta]$ and s_0 data in terms of Yamakawa et al.'s theories[2] for rigid cylinders yielded 0.30 ± 0.01 nm and 2.6 ± 0.4 nm for the contour length per main chain residue and diameter of the schizophyllan trimer, respectively. From the agreement of these values with the pitch (per residue) and diameter of the model triple helix for schizophyllan, it was concluded that this polysaccharide dissolves in water as a rigid triple helix. The rigidity of this helix expressed in terms of the experimentally determined persistence length (200 nm) was comparable to that of native collagen,[3] a triple helical biopolymer.

Chapter III. Dissociation of the Schizophyllan Triple Helix

Schizophyllan in water-DMSO mixtures at 25°C and in pure water at temperatures from 20 to 160°C were investigated by viscosity, optical rotatory dispersion, and viscoelasticity. The intrinsic viscosity for a given sample in water-DMSO mixtures at 25°C almost discontinuously decreased to the value in pure DMSO, when the weight fraction of DMSO in the mixture was increased to 87 %. This finding indicates that the schizophyllan triple helix in aqueous DMSO 'melts' abruptly to three single chains at the critical DMSO composition of 87 %. When a pure water solution was heated, the triple helix also melted to single chains at about 135°C. However, the triple helix was not recoverable once separated to single chains in DMSO at 25°C or in water at temperatures above 135°C.

Chapter IV. Correlation between the Antitumor Activity of Schizophyllan and its Triple-Helical Conformation in Dilute Aqueous Solution

Low molecular weight samples of schizophyllan

ranging in M_w(in water) from 5×10^3 to 1.3×10^5 were prepared by extensive sonication, and their antitumor activities (expressed in terms of the tumor inhibition ratio) against Sarcoma 180 ascites, $[\eta]$, and gel-filtration chromatograms in aqueous solution were determined.

The tumor inhibition ratio was essentially unity for M_w higher than 9×10^4, but decreased to zero or even became negative for M_w lower than 10^4. Combination of $[\eta]$ and chromatographic data showed that above $M_w \sim 9 \times 10^4$ the predominant species of schizophyllan in aqueous solution is the rigid triple helix, whereas below $M_w \sim 9 \times 10^4$ both triple helices and single chains coexist in the solution and the fraction of the former decreases monotonically to zero with decreasing M_w to 5×10^3. Therefore, we concluded that the antitumor potency of schizophyllan in water is related to the amount of triple helices relative to that of coexisting single chains.

Chapter V. Triple Helix and Random Coil of Polytran Scleroglucan in Dilute Solution

A series of native and sonicated samples of Polytran

scleroglucan were investigated by chemical analysis as well as by light scattering, viscosity, and ultracentrifugation in water containing 0.01 N NaOH, DMSO, and water-DMSO mixtures at 25°C.

From the chemical analysis, this scleroglucan was found to be a β-1,3-D-glucan consisting essentially of the same repeating units as those of schizophyllan. Data for $\langle S^2 \rangle^{1/2}$, $[\eta]$, and s_0 as functions of M_w, combined with those for the ratio M_w(in 0.01 N NaOH)/ M_w(in DMSO), showed that Polytran scleroglucan dissolves in DMSO as single randomly coiled chains very similar to those of schizophyllan in the same solvent, while it dissolves in 0.01 N NaOH as rodlike trimers or higher aggregates, depending on whether M_w(in DMSO) is lower or higher than 2×10^5.

From $\langle S^2 \rangle^{1/2}$, $[\eta]$, and s_0 for samples with M_w(in DMSO) $\lesssim 1 \times 10^5$ in 0.01 N aqueous NaOH, the contour length per main chain residue and the diameter of the scleroglucan trimer rod were found to be 0.30 ± 0.03 and 2.6 ± 0.5 nm, respectively, which agree with the pitch and diameter of the schizophyllan triple helix. The $[\eta]$ values of sonicated samples in water-DMSO mixtures almost discontinuously decreased at about

87 % DMSO at which the schizophyllan triple helix dissociates to single chains. On the basis of these results and structure information, it was concluded that the scleroglucan trimer has essentially the same triple helical structure as that of schizophyllan. Evidence was obtained for higher aggregates [for M_w(in DMSO) > 2×10^5] in 0.01 N aqueous NaOH being built up of triple-helical trimers.

Chapter VI. Cholesteric Mesophase in Aqueous Solutions of Polytran Scleroglucan

Concentrated aqueous solutions of two scleroglucan samples with M_v(in water) of 1.93×10^5 and 1.24×10^5 were studied by polarizing microscopy and ORD at temperatures from 11 - 35°C.

Birefringent phases appeared in the continuous isotropic phase at w ∿ 0.14 and 0.19 for the higher and lower molecular weight samples, respectively, and spread over the entire solution at w ∿ 0.19 and 0.31, almost independently of temperature. Microscopic patterns of these birefringent phases resembled those reported for cholesteric liquid crystals.[4] The $[\alpha]_{436}$ at 21.5°C markedly increased with increasing w between 0.14 and

0.19 and leveled off at a value about 70 times the infinite dilution value. From these findings, the birefringent phase of aqueous Polytran scleroglucan was concluded to be cholesteric. The intrinsic birefringence of the scleroglucan triple helix in the cholesteric solution was estimated from optical rotation data to be $(1.5 \pm 0.5) \times 10^{-2}$.

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2. ref. 2 - 5 in Chapter II.
3. ref. 15 in Chapter II.
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LIST OF SYMBOLS

P: cholesteric pitch 142

P(θ): intramolecular scattering function 93

Q: apparent value of M_w/M_z at finite polymer concentration 19

Q(θ): intermolecular scattering function 93

R: gas constant 18

R_{90,U_u}: Rayleigh ratio of benzene at 90° for unpolarized light 92

R_θ: reduced scattering intensity ($\equiv R_{\theta,U_v}$) 28

R_{θ,U_v}: R_θ 93

S: distance between successive parallel lines in fingerprint patterns 133

$\langle S^2 \rangle$: mean-square radius of gyration 25

T: temperature 18

V: velocity of a rolling ball 51

c: polymer mass concentration 21

$\bar{c}$: mean polymer mass concentration at sedimentation equilibrium 18

c_0: initial polymer mass concentration in a sedimentation equilibrium experiment 18

c_a: equilibrium polymer mass concentration at the meniscus of liquid column 18

c_b: equilibrium polymer mass concentration

at the bottom of liquid column 18

d: diameter of a polymer chain (Chapters II and V); thickness of a rectangular ORD cell (Chapter VI) 36;129

f: weight fraction of trimers 74

h: contour length per main chain residue 39

k': Huggins' constant 29

k_s: constant in $s = s_0/(1 + k_s c)$ 23

n_0: refractive index of solvent 93

n_b: refractive index of benzene 92

n_α: principal refractive index in the longitudinal direction of a cylindrical molecule 144

n_γ: principal refractive index in the transverse direction of a cylindrical molecule 144

$n_\parallel$: refractive index of a cholesteric layer in the longitudinal direction of director 142

$n_\perp$: refractive index of a cholesteric layer in the transverse direction of director 142

Δn: excess refractive index (Chapters II and V); cholesteric layer birefringence (Chapter VI) 21;142

$\partial n/\partial c$: specific refractive index increment 22

q: persistence length 40

r_a: radial distance from the center of rotation to the meniscus of a cell 18

r_b: radial distance from the center of rotation to the bottom of a cell 18

r_p: radial distance from the center of rotation to the pesk position 22

s: sedimentation coefficient 22

s_0: limiting sedimentation coefficient 23

t: time 23

Δt_p: duration of preheating a solution 60

$\bar{v}$: partial specific volume of polymer 18

w: polymer weight fraction 129

w_c: average tumor weight of glucan-untreated groups of mice 7

w_t: average tumor weight of glucan-treated groups of mice 7

x: concentration of sodium hydroxide 120

Γ: molecular weight ratio of M_w(in water) to M_w(in DMSO) 73

Θ: optical rotation angle per unit length of cholesteric liquid crystal 142

Φ: volume fraction of isotropic phase 139

Ψ: instrument constant of light scattering photogoniometer 91

α: linear expansion coefficient of glass 51

$[\alpha]_\lambda$: specific rotation at wavelength λ 54

β: Flory-Scherage-Mandelkern parameter 42

δ: twisted small angle between successive cholesteric thin layers 146

$\zeta(\phi)$: parameter characterizing the degree of ordering of cylindrical molecules 144

η: viscosity coefficient 50

$[\eta]$: intrinsic viscosity 8

η_0: solvent viscosity 38

η_r: relative viscosity 50

η_{sp}: specific viscosity 122

θ: scattering angle 28

λ: wavelength 55

ξ: tumor inhibition ratio 6

ρ: polymer solution density 20

ρ_0: solvent density 18

ρ_b: density of glass 51

ρ_u: depolarization ratio 92

ϕ: volume fraction of polymer 129

ψ: inclination angle of a rolling-ball viscometer 51

ω: angular velocity of a rotor 18

LIST OF PUBLICATIONS

Part of this thesis has been or will be published in the following papers.

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