Polymer Journal, Vol. 14, No. 2, pp 93—99 (1982)

Study on Polysaccharide by the Fluorescence Method. II. Micro-Brownian Motion and Conformational Change of Amylose in Aqueous Solution

Shinichi KITAMURA, Harunobu YUNOKAWA, Sanae MITSUIE, and Takashi KUGE

Laboratory of Biopolymers, Department of Agricultural Chemistry, Kyoto Prefectural University, Sakyo-ku, Kyoto 606, Japan.

(Received March 23, 1981)

ABSTRACT: The segmental motion of amylose in aqueous solution and the conformational transition with temperature and pH were studied by the fluorescence polarization method. We prepared two types of fluorescent conjugates of amylose: amylose conjugated with fluorescein randomly throughout chain (F-Amylose) and amylose conjugated locally on a terminal segment (*t*-F-Amylose). The value of the rotational relaxation time for *t*-F-Amylose was much smaller than that for F-Amylose. This result suggests that a terminal segment undergoes a more rapid micro-Brownian motion than interior segments. Polarization curves of these conjugates gradually departed from the Perrin equation when the temperature was raised. F-Amylose showed a gradual departure above 45°C but *t*-F-Amylose showed a departure at room temperature. This indicates that the conformational transition occurs first in terminal segments and then extends to the interior segments with increasing temperature. The effect of pH on the polarization was also examined. When measured as a function of pH, fluorescence depolarization increased pronouncedly in the alkaline region. In contrast to changing temperature, depolarization began to increase at the same pH for the two amylose conjugates.

KEY WORDS Amylose / Fluorescent Conjugate / Fluorescence Depolarization / Segmental Motion / Terminal Segment / Interior Segment / Deformed Helix / Conformational Transition /

The conformation of amylose in an aqueous solution has been intensively studied by means of hydrodynamic methods.[1-4] These studies suggest that amylose behaves as a random coil in an aqueous solution. It is therefore considered that an amylose chain takes on many different shapes and fluctuates continuously among these. However, we note that hydrodynamic measurements can provide information only about the overall character of the molecule. The local conformation of amylose in an aqueous solution is a matter of dispute in spite of extensive investigations.[5,6]

The fluorescence polarization method is expected to provide information on the segmental motion and the conformation of a polymer chain to which a fluorescent residue is attached. Thus, when a polymer chain undergoes a conformational transition, the course of the transition may be followed by measuring the fluorescence polarization of the attached dye residue.[7-9]

The purpose of the present study is twofold: to obtain information on segmental motion and the conformation of amylose about the attached dye residue and to study the conformational transition of amylose induced by temperature and pH changes, both by the fluorescence polarization method. We prepared two types of fluorescent conjugates of amylose: amylose conjugated randomly with fluorescein residues throughout the chain and amylose conjugated only at a terminal segment. These fluorescent conjugates enabled us to investigate the differences in segmental mobility and conformation between the interior and terminal segments of amylose.

EXPERIMENTAL

Basic Principle of Synthetic Amylose Preparation

Phosphorylase catatyzes the reaction represented by,

$$n\cdot(\text{glucose 1-phosphate})+\text{primer} \rightleftarrows \text{amylose}+n\cdot(\text{orthophosphate})$$

When glucose 1-phosphate is in excess, the forward reaction occurs predominantly. It is well known that this synthetic reaction takes place in a mode all the primer chains grow at an approximately equal rate.[10] Hence, one product of this reaction may be nearly monodisperse synthetic amylose. The molecular weight of the product can be calculated from the amount of phosphate liberated in the reaction mixture and the concentration of the primer used.

Preparation of F-Amylose

Maltopentaose was obtained by fractionation of acid hydrolyzates of β-cyclodextrin by cellulose column chromatography. The potato phosphorylase used was a gift from Professor Toshio Fukui of Osaka University. The enzyme was free from other carbohydrase activity.[11] The reaction mixture (4×10^{-5} M maltopentaose, 0.1 M glucose 1-phosphate, 120 units of phosphorylase and 0.01 M maleate buffer in total volume of 200 ml) was incubated at 35°C for 450 min. The reaction was stopped by the addition of 100 ml of 5% trichloroacetic acid, followed by neutralization with KOH. This reaction mixture was dialyzed against dimethylsulfoxide (DMSO)–water (1 : 4, v/v) solution at 35°C for 2 days. After dialysis, 250 ml of ethanol was added to precipitate the amylose produced. The precipitate was washed five times with 500 ml of ethanol, twice with 300 ml of ether and dried overnight *in vacuo* at 70°C. The amylose prepared by this procedure had a number-average molecular weight of 110,000. The amylose was shown to be nearly monodisperse by gel permeation chromatography using a Sepharose CL-4B (Pharmacia, Uppsala).

Conjugation with fluorescein was carried out according to the method of Belder described previously.[7,12] The degree of substitution with dye was regulated by changing the amount of dye added to the reaction mixture. The conjugate obtained, F-Amylose, is considered to have fluorescein residues randomly on the chain.

Preparation of t-F-Amylose

Using the maltopentaose-fluorescein conjugate as a primer in the synthetic reaction catalyzed by phosphorylase, we obtained amylose specifically modified with a fluorescein residue near the chain end. The name of this type of conjugate is *t*-F-Amylose. A reaction mixture (2.6×10^{-5} M fluorescein-maltopentaose conjugate, 0.1 M glucose 1-phosphate, 30 units of phosphorylase and 0.01 M maleate buffer in total volume of 50 ml) was incubated at 35°C for 300 min. The enzymatic reaction was stopped by dialysis. Thereafter, the reaction mixture was dealt with in the same way as F-Amylose.

Elution pattern of t-F-Amylose by Sepharose CL-4B

Gel permeation chromatography of *t*-F-Amylose was performed using a Sepharose CL-4B in order to investigate molecular weight distribution. The chromatographic column used was 70 cm long and 2.1 cm in diameter. The elution solvent was 0.05 M aqueous KOH solution. The flow rate was regulated to 0.33 ml min^{-1}. The parameters V_0 and V_t were determined using blue dextran (Pharmacia, Uppsala) and KCl, respectively. 8 mg of *t*-F-Amylose were dissolved in 0.5 ml DMSO, diluted to 5 ml with distilled water, and 2 ml of this solution were charged on the column. Each 5 ml of eluent was collected. The carbohydrate content in each fraction was determined by the phenol-sulfuric acid colorimetric method[13] and fluorescent intensity at 520 nm was measured using a Shimadzu spectrofluorometer RF-502. For calibration of the column, a series of synthetic amylose with molecular weights of 0.88, 2.6, 5.8, 11, 13, 36, and 99×10^4 were used.

The elution pattern of *t*-F-Amylose is shown in Figure 1. Two peaks are seen at V_0 and 150 ml on the total carbohydrate curve, but only one peak at 150 ml in the fluorescence intensity curve. The carbohydrate which collected about V_0 exhibited virtually no fluorescence and may have been synthesized from maltopentaose without fluorescein. The molecular weight of the amylose conjugated with fluorescein was estimated to be 130,000 from the elution volume for the maximum fluorescence intensity.

Methods

Fluorescence polarization and fluorescence life-

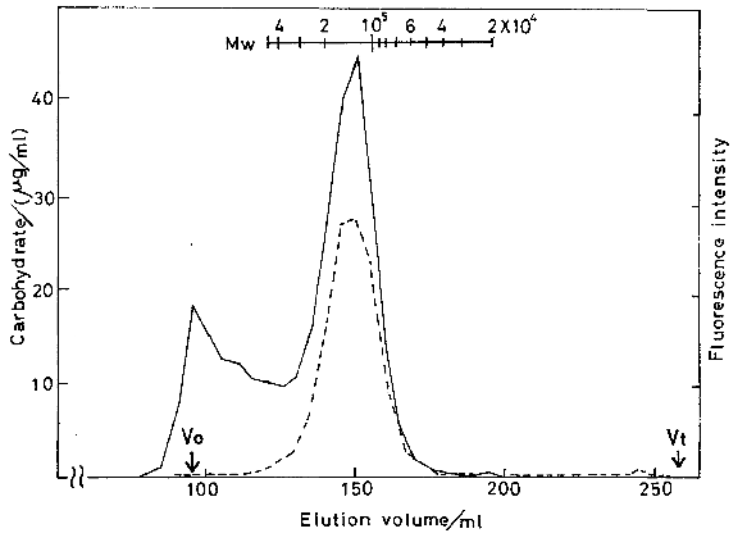

Figure 1. Elution pattern of *t*-F-Amylose by GPC using Sepharose CL-4B. (—), carbohydrate; (---), fluorescence intensity.

time measurements were performed as described before.[7]

The mean rotational relaxation time, $\langle\rho\rangle$, of the kinetic unit carrying the emission oscillator was computed from an equation of the Perrin type,[7,14]

$$\frac{1}{P}-\frac{1}{3}\approx\left(\frac{1}{P_0'}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\langle\rho\rangle}\right) \qquad (1)$$

where P_0', τ and $\langle\rho\rangle$ have the same meaning as in the previous paper.[7]

Since F-Amylose and *t*-F-Amylose were insoluble in water, they were dissolved in a small amount of DMSO and diluted with a phosphate buffer solution (0.01 M, pH 7). The polymer concentrations of the final solutions were from 0.015—0.08%. In the pH profile experiments, dilution was performed with distilled water and the pH of the solution was adjusted by adding an aqueous KOH solution of appropriate concentration. The viscosities of water and sucrose solution were taken from International Critical Tables[15] and Kagaku Binran.[16]

RESULTS

Some Properties of F-Amylose and t-F-Amylose

The absorption spectra of F-Amylose and *t*-F-Amylose were similar to that of fluorescein except for a red shift from 2 to 4 nm. The emission spectra of these conjugates were also similar to that of fluorescein except for a red sift of about 5 nm. The degree of substitution of dye was estimated spectrophotometrically using methyl *N*-fluoresceinlythiocarbamate as a reference. The degree of substitution for *t*-F-Amylose in Table I was estimated, excluding amylose collected about V_0 in Figure 1. The degrees of substitution for F-Amylose and *t*-F-Amylose were essentially the same. These conjugates have nearly the same fluorescence lifetimes as that of the free dye. The results are summarized in Table I.

Comparison of $\langle\rho\rangle$ values for F-Amylose and t-F-Amylose

Figure 2 shows plots of $1/P$ *versus* T/η for F-Amylose and *t*-F-Amylose at 25°C. The solvent viscosity was varied by the addition of sucrose. Since P_0' can be estimated from the ordinate intercept obtained by linear extrapolation, the value of $\langle\rho\rangle/\tau$ for each conjugate can be calculated by eq 1. Combining this value with the fluorescence lifetime, we can calculate $\langle\rho\rangle$. Table II shows the values of $1/P_0'$, $\langle\rho\rangle/\tau$, τ, and $\langle\rho\rangle$ for F-Amylose and *t*-F-Amylose. The $\langle\rho\rangle$ value for F-Amylose is nearly twice as large as that for *t*-F-Amylose. Therefore, it may be said that the terminal segments

Table I. Some properties of F-Amylose and *t*-F-Amylose[a]

Conjugate	Molecular weight	DS	Wavelength/nm		Fluorescence lifetime/ns
			Absorption maximum	Emission maximum	
Fluorescein	376	—	491	513	4.3
F-Amylose	110000	0.001	495	518	4.4
t-F-Amylose	130000	0.0009	493	519	4.5

[a] DS, degree of substitution with dye is defined as the fraction of glucose residues substituted. Absorption and emission spectra, and fluorescnece lifetimes were measured at pH 7, 0.01 M phosphate buffer solution.

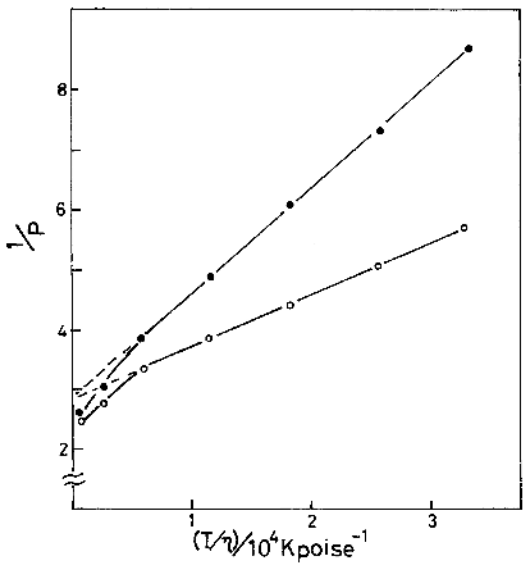

Figure 2. Plots of $1/P$ *vs.* T/η for F-Amylose (○) and *t*-F-Amylose (●) in 0.01 M phosphate buffer solution of pH 7 at 25°C. The solvent viscosity was varied by the addition of sucrose.

show more rapid segmental motion than the interior segments.

Effect of Temperature on the Polarization

Figure 3 shows the effect of temperature on the polarization of F-Amylose and *t*-F-Amylose. It is seen that significant upward curvatures are present in these Perrin plots. This behavior on the part of F-Amylose is attributed to the accelerated loosening of the internal structure of the amylose chain with an increase in temperature.[7] Although for F-Amylose the upward curvature is seen at temperatures above about 45°C, *t*-F-Amylose begins to show the same behaivor at as low a temperature as 25°C.

Table II. Comparison of $1/P_0'$, $\langle\rho\rangle/\tau$, τ, and $\langle\rho\rangle$ values for F-Amylose and *t*-F-Amylose in a 0.01 M phosphate buffer solution at 25°C

Conjugate	$1/P_0'$	$\langle\rho\rangle/\tau$	τ/ns	$\langle\rho\rangle$/ns
F-Amylose	2.79	2.54	4.4	11
t-F-Amylose	2.90	1.35	4.5	6.1

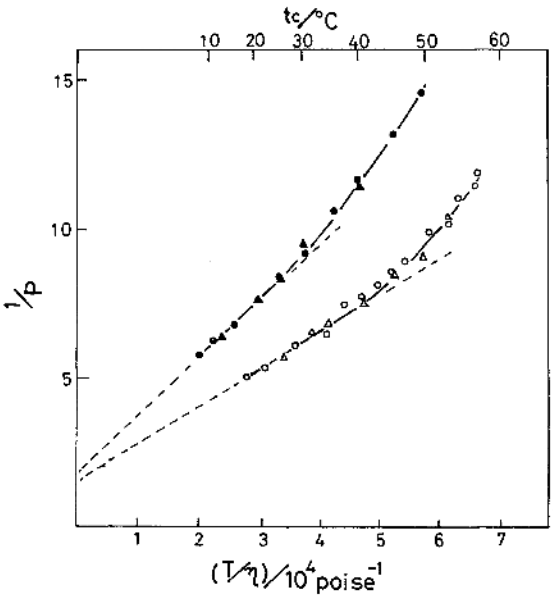

Figure 3. Effect of temperature on the polarization of F-Amylose (○, △) and *t*-F-Amylose (●, ▲). The temperature increased (○, ●) and subsequently decreased (△, ▲).

This difference suggests that the conformational transition of amylose first occurs in the terminal segments and then extends to all segments with increasing temperature.

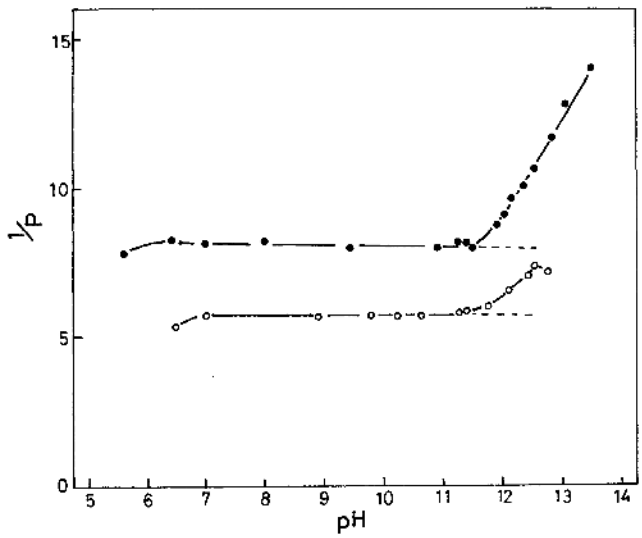

Figure 4. Effect of pH on the polarization of fluorescence for F-Amylose (○) and *t*-F-Amylose (●) at 25°C.

Effect of pH on the Polarization

Figure 4 shows the pH dependence of fluorescence polarization of F-Amylose and *t*-F-Amylose. The $1/P$ values for these conjugates stay constant at a pH below 11.5, but above this, they increase with increasing pH. The fluorescence lifetimes of the conjugates in the alkaline region above pH 12 were found experimentally to be essentially the same as those in a neutral solution.

It has been shown that amylose in an aqueous solution exhibits considerable change in intrinsic viscosity and optical rotation in the region of pH between 11 and 13, and this behavior has been interpreted in terms of the conformational change caused by increasing negative charges on the polymer chain.[17-19] It can be concluded that the increase in the depolarization observed here is caused by the same conformational transition. In contrast to the temperature dependence, the transition of either conjugate becomes appreciable at nearly the same pH.

DISCUSSION

It was found in this study that the terminal segment of an amylose chain undergoes more rapid micro-Brownian motion than the interior segment. Uchida *et al.* reported a similar finding for polystyrene.[20] These results are reasonable since the motion of terminal segments is restricted only by the linkage on one side, but the motion of the interior segments is restricted by linkages on both sides. The interior segments of the chain must restrict their motion as in the case of "crankshaft-like motion"[21,22] and others.[23,24]

The results shown in Figures 4 and 5 indicate that amylose in neutral aqueous solutions has more or less an ordered conformation (helix) with restricted rotation around glucoside linkage at room temperature. The ordered conformation of amylose in solution has been interpreted in terms of the "deformed helix,"[17,25,26] "interrupted helix"[5,27,28] or their modified models.[29,30] According to the interrupted helix model, tight helical segments of the chain are essentially identical to the helix of "V" amylose in the solid state. If tight helical segments having a DP 120 exist in an amylose chain as suggested by Szejtli *et al.*,[28] we might expect a large rotational relaxation time as shown from the following calculations.

Consider an amylose molecule composed of tight helical segments, each containing 120 glucose residues with a completely flexible chain between them. The flexible chain is assumed not to restrict the motion of the helical segments. If each helical segment consists of 15 helical turns, *i.e.*, eight glucose units per turn, the rotational relaxation time for each segment would be equivalent to that for a

long cyclindrical rod of 150 Å length and 18 Å diameter. A long cylindrical rod, of length L and diameter d, can be approximated by an prolate ellipsoid of the same volume and length ($2a=L$) with semi-axes a and b satisfying the following equation,[31]

$$\frac{b}{a}=\left(\frac{3}{2}\right)^{1/2}\frac{d}{L} \tag{2}$$

The harmonic mean of the two principal relaxation times of rotation is given by,

$$\rho_h=\frac{3}{\left(\frac{1}{\rho_a}+\frac{2}{\rho_b}\right)} \tag{3}$$

where ρ_a and ρ_b are the relaxation times of the axis of revolution and that perpendicular to it, respectively. ρ_a and ρ_b are calculated from,[32]

$$\frac{\rho_a}{\rho_0}=\frac{2}{3}\times\frac{1-(b/a)^4}{[2-(b/a)^2]\dfrac{(b/a)^2}{\sqrt{1-(b/a)^2}}\ln\dfrac{1+\sqrt{1-(b/a)^2}}{(b/a)}-(b/a)^2} \tag{4}$$

$$\frac{\rho_b}{\rho_0}=\frac{4}{3}\times\frac{1-(b/a)^4}{[1-(b/a)^2]\dfrac{(b/a)^2}{\sqrt{1-(b/a)^2}}\ln\dfrac{1+\sqrt{1-(b/a)^2}}{(b/a)}+1} \tag{5}$$

$$\rho_0=\frac{1}{kT}4\pi ab^2 \tag{6}$$

where ρ_0 is the rotational relaxation time of the sphere having the same volume as the ellipsoid and k is the Boltzmann constant. With these equations, the ρ_h for the helical segment in water at 25°C is estimated to be 43 ns. This may be the smallest value that can be expected theoretically, since completely free linkage is assumed between the tight helical segments. However, only about a quarter of this value was found experimentally (Table II). This fact suggests that amylose does not take on a tight helical conformation in an aqueous solution.

The "deformed helix" and its modified conformations proposed by several authors for amylose in an aqueous solution can be approximated as those which somewhat resembling wormlike coils consisting essentially of an imperfect or deformed helical backborne.[17,25,26,29,30] We consider that the increased segmental motion increases the imperfectness of the helical backborne and promotes a random-coil character of the amylose molecule throughout the chain. The "deformed helix" is thus considered to be more reasonable as a model for the conformation of amylose in an aqueous solution than "interrupted helix."

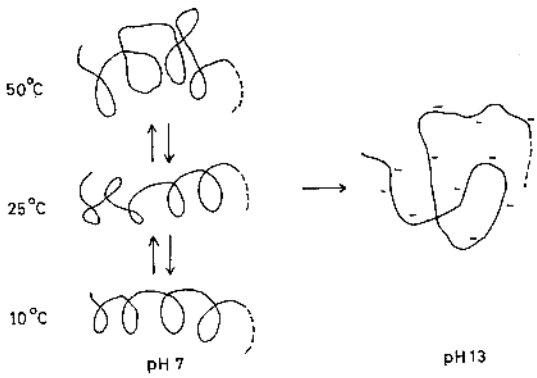

Figure 5. Model proposed for the changes in the conformation of amylose with temperature and pH.

We propose a molecular mechanism illustrated schematically in Figure 5 in order to interpret the conformational change revealed by the fluorescence polarization data. The loosening of the helical structure with increasing temperature may occur first in the terminal segments at room temperature and then extend to all segments above 45°C. In contrast to the loosening caused by thermal energy, the loosening in alkaline solution occurs in the all segments at the same pH due to the increased electrostatic repulsion between dissociated hydroxyl groups of glucose units.

Acknowledgements. The authors should like to thank Professor Y. Nishijima of Kyoto University for perimission to use the nanosecond fluorometer and also thank Dr. S. Ito for his help in performing the fluorescence lifetime measurements. Appreciation is due to Associate Professor M. Yamamoto of Kyoto University for his useful comments and criticism. We are indebted to Professor T. Fukui of Osaka University for his generous gift of potato phosphorylase.

REFERENCES

1. J. M. G. Cowie, *Makromol. Chem.*, **42**, 230 (1961).
2. W. Burchaurd, *Makromol. Chem.*, **64**, 110 (1963).
3. W. Banks and C. T. Greenwood, *Makromol. Chem.*, **67**, 49 (1963).
4. W. Banks and C. T. Greenwood, *Carbohydr. Res.*, **7**, 349 (1968).
5. W. Banks and C. T. Greenwood, "Starch and Its Components," Edinburgh University Press, Edinburgh, U.K., 1975, Chapter 4.
6. T. Kuge, "Denpun Kagaku Handbook," N. Nakamura and S. Suzuki, Ed., Asakura Shoten Co., Tokyo, 1977, pp 20—23.
7. S. Kitamura, H. Yunokawa, and T. Kuge, *Polym. J.*, **14**, 85 (1982).
8. E. V. Anufrieva, M. V. Volkenstein, M. G. Krakovyak, and T. V. Shevelava, *Dokl. Akad. Nauk. SSSR*, **186**, 854 (1969).
9. T. J. Gill III and G. S. Omenn, *J. Am. Chem. Soc.*, **87**, 4188 (1965).
10. W. J. Whelan and J. M. Bailey, *Biochem. J.*, **58**, 560 (1954).
11. A. Kamogawa, T. Fukui, and Z. Nikuni, *J. Biochem.*, **63**, 361 (1968).
12. A. N. De Belder and K. Granath, *Carbohydr. Res.*, **30**, 375 (1973).
13. J. E. Hodge and B. T. Hofreiter, "Method in Carbohydrate Chemistry I," R. L. Whistler and M. L. Wolfrom, Ed., London, U.K., 1962, p 388.
14. G. Weber, *Biochem. J.*, **51**, 145 (1952).
15. "International Critical Tables," Vol. V, E. W. Washburn, Ed., McGraw-Hill, 1929, p 23.
16. "Kagaku Binran," Vol. 1 (II), The Chemical Society of Japan, Ed., Maruzen Co., Tokyo, 1966, Sections 5—10.
17. V. S. R. Rao and J. F. Foster, *Biopolymers*, **1**, 527 (1963).
18. L. Doppert and A. J. Staverman, *J. Polym. Sci., A-1*, **4**, 2373 (1966).
19. S. R. Erlander, R. M. Purvinas, and H. L. Griffin, *Cereal Chem.*, **45**, 140 (1968).
20. M. Uchida, M. Yamamoto, and Y. Nishijima, *Polym. Prepri. Jpn.*, **25**, 2. 297,298 (1976).
21. T. F. Schatzki, *J. Polym. Sci.*, **57**, 496 (1962).
22. E. Helfand, *J. Chem. Phys.*, **54**, 4651 (1971).
23. B. Valeur, J. P. Jarry, F. Gény, and L. Monnerie, *J. Polym. Sci., Polym. Phys. Ed.*, **13**, 667 (1975).
24. F. Gény and L. Monnerie, *J. Polym. Sci., Polym. Phys. Ed.*, **17**, 131 (1979).
25. T. Kuge and S. Ono, *Bull. Chem. Soc. Jpn.*, **34**, 1264 (1961).
26. T. Kuge, *Bull. Univ. Osaka Pref., B*, **11**, 121 (1961).
27. J. Holló and J. Szejtli, *Stärke*, **10**, 49 (1958).
28. J. Szejtli, M. Richter, and S. Augustat, *Biopolymers*, **5**, 5 (1967).
29. M. Senior and E. Hamori, *Biopolymers*, **12**, 65 (1973).
30. R. C. Jordan, D. A. Brant, and A. Cesáro, *Biopolymers*, **17**, 2617 (1978).
31. C. Tanford, "Physical Chemistry of Macromolecules," John Wiley & Sons, Inc., New York–London, 1961, p 343.
32. F. Perrin, *J. Phys. Radium*, **5**, 497 (1934).