Change in Molecular Weight of Hyaluronic Acid During Measurement with a Cone-Plate Rotational Viscometer

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ABSTRACT: It was shown using a cone-plate rotational viscometer that the apparent viscosity of a dilute aqueous solution of sodium hyaluronate decreased gradually during the measurement. Hyaluronic acid (HA) forms a characteristic network by entanglements coupling, so two hypotheses could be postulated from the reduction of viscosity due to shearing stress. One was that disentanglement of the temporary network occurred, and the other was that scission of HA chains was responsible. In this study, the reason for the reduction in viscosity was clarified using high-performance gel permeation chromatography with low-angle laser light scattering. It was thus demonstrated that chain scission occurred during the viscometric measurement. On the assumption that the HA degradation was caused by shearing stress, the effects of shearing rate, the initial molecular weight, salt, and polymer concentrations of samples were investigated. High molecular weight HA chains were preferably severed. From the change in polydispersity of the samples it was inferred that due to the viscoelasticity of HA solution scission behavior would differ, depending on the shearing rate. Also, the salt and polymer concentrations were found to exert large influences on the degradation. Thus, it was concluded that the scission rate was related to the expansion of HA molecules in solution. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 67: 2199–2206, 1998

Key words: hyaluronic acid; degradation; rotational viscometer; molecular weight; gel permeation chromatography with low-angle laser light scattering

INTRODUCTION

Hyaluronic acid (HA) is a mucopolysaccharide with a molecular weight of 10^4–10^7 noted for possessing viscoelasticity. This property is due to the features of HA structure that it is fundamentally a network-forming polymer.[1–4] By means of rotary shadowing electron microscopy, Scott et al. demonstrated that HA chains formed an irregular honeycomb-like structure of enormous dimensions even at the relatively low concentration of 1 μg/mL.[1] This is considered to be the reason for the unusual high viscoelasticity of the solution. The HA network construction can be enhanced or reduced quite easily under the influence of physical and chemical factors. Such variables as molecular weight,[1,3,4] concentration,[1,2,4] and other supplementations[4] affect the capacity for network formation. If this construction is weakened, the viscosity will decrease in spite of a constant molecular weight of the HA chains.

With respect to biological and synthetic polymers, numerous earlier observations of depolymerization induced by physically applied energy, such as with high shear stirring,[5,6] laminar[7,8] or capillary flow,[9–12] and radiation[13,14] are scattered through out the literature. By subjecting polymers to sufficiently vigorous mechanical energy, ruptures of the chemical bonds in some polymer molecules are achieved. In most of the reported

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studies, the degradation was followed by the fluidity of the solutions. During measurement with a cone-plate rotational viscometer, we noted a decrease in the viscosity of HA solutions. Two hypotheses can be postulated regarding the mechanism of such reduction. In the first, the polymer chains disentangled by the shearing stress and oriented so that flow was facilitated, while the lengths of the chains presumably remained constant. In the second, the polymer chains themselves were shortened, this causing conformational change in the secondary or tertiary structures to reduce the viscosity. If the former was the case, the change in viscosity could not be used as a parameter for the degree of degradation. To clarify the cause of reduction in viscosity, we therefore observed changes in HA molecular weight directly, using high-performance gel permeation chromatography (GPC) with low-angle laser light scattering (LALLS). As a result, we obtained evidence that shearing stress caused scission of HA molecular chains.

MATERIALS AND METHODS

Materials

Two HA samples (sodium salts) with weight average molecular weight (M_w) of about 2.6×10^6 and 1.1×10^6 were used. The high molecular weight HA (HA-A) extracted from culture medium of *Streptococcus equi* was supplied by Denki Kagaku Kogyo Co., Ltd. The low molecular weight HA (HA-B) isolated from rooster comb was supplied by Seikagaku Kogyo Co., Ltd. To avoid chain scission by magnetical stirring, HA solutions were prepared by gentle tumbling in the refrigerator after sufficient swelling at ambient temperature. All the water used was deionized and then Millipore filtered. Other chemicals were commercial reagent grade.

Shearing Apparatus

A cone-plate rotational viscometer of E type made by Tokimec Co., Ltd. was used as the shearing apparatus. The diameter and angle of the cone were 24 mm and 1° 34′, respectively. Shearing was carried out at 37.0°C, controlled with circulated water. The load was controlled by changing the shearing rate and the run-time period.

GPC-LALLS

GPC-LALLS was performed on three columns, a TSK-guard column $\mathrm{PW_{XL}}$ (6.0×40 mm), a TSK-$\mathrm{G6000PW_{XL}}$ (7.8×300 mm), and a TSK-$\mathrm{G3000PW_{XL}}$ (7.8×300 mm). The mobile phase was $0.2M$ aqueous NaCl solution with the flow rate of 0.5 mL/min. Samples were diluted by mobile phase into about 0.02% at final concentration and 400 μL portions were injected. Peak elutions were monitored with a LALLS photometer (Tosoh LS-8000) and a differential refractometer (Tosoh RI-8012, RI). The values of molecular weight and polydispersity were estimated using the Tosoh GPC-LALLS data processing program with non-approximation in the LALLS mode.

RESULTS AND DISCUSSION

Falloff of Apparent Viscosity of HA Solution

A solution of HA is said to be a typical non-Newtonian fluid, a pseudoplastic solution. As non-Newtonian fluids show different viscosity values according to the shearing rate and shearing stress, values measured at various shearing rates are often used for analysis of flow curves. With pseudoplastic solutions, double logarithmic plots of apparent viscosity vs. shearing rate commonly indicate downward linear relationships. In the present study, the measured viscosity values of 0.1% HA in $0.2M$ NaCl solution decreased gradually with no change of sample in the cup of the viscometer (Fig. 1). It was doubted that the thixotropic phenomenon would occur due to disentanglement of the temporary network, but after a long period of standing without shearing, the reduction in viscosity did not recover. The decrease in apparent viscosity was more remarkable in HA-A, with high molecular weight, than HA-B, with low molecular weight.

HA Degradation During Viscometric Measurement

To clarify whether the falloff of apparent viscosity was due to disentanglement of the network by shearing stress or to degradation of polymer chains, the molecular weights of the HA samples were observed before and after viscometric measurement. Samples were removed and subjected to GPC-LALLS after each measurement run. The results revealed a gradual decrease in M_w, especially in the case of HA-A (Table I). To determine the M_w during the course of a run, it was neces-

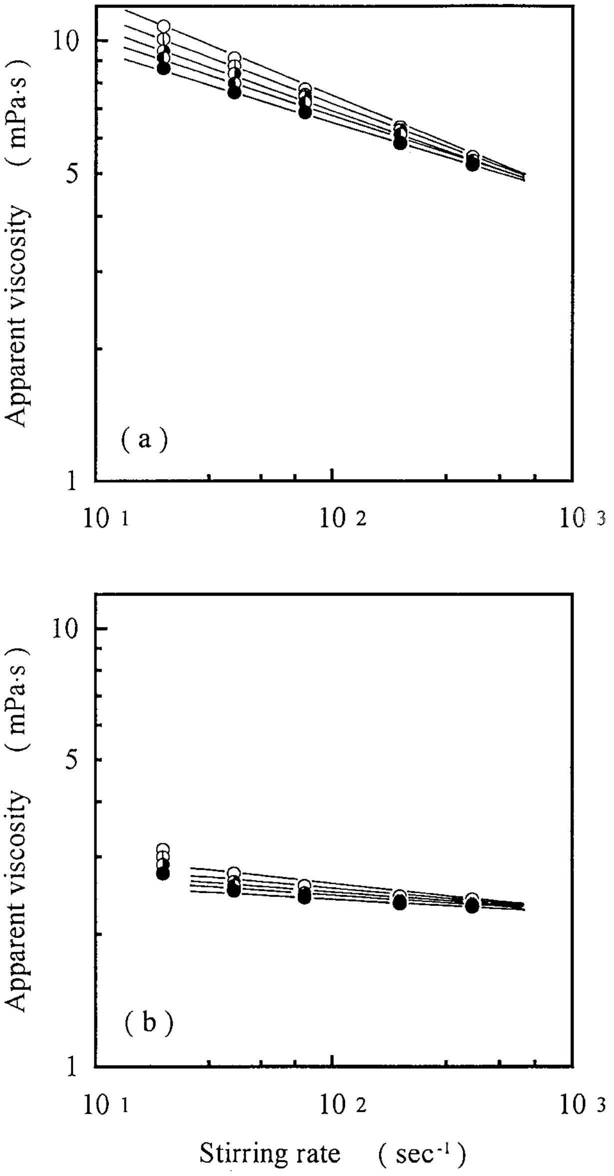

Figure 1 Changes in apparent viscosity of HA solutions. Measurement was performed at 37°C. The shear rate was increased from 0.5 rpm to 100 rpm at 3-min intervals. (a) 0.1% HA-A in 0.2*M* NaCl; (b) 0.1% HA-B in 0.2*M* NaCl: (○) 1st; (◐) 2nd; (◑) 3rd; (◐) 4th; (●) 5th measurement.

sary to sacrifice the run at that point, so the values listed in the table were not obtained consecutively in one viscometric measurement. The values were averages from at least four estimations. Initially HA-A had a M_w of about 2.57 × 10^6, but this decreased gradually with each run, and after the fifth measurement it was reduced to approximately 70% of the initial value. On the other hand, in the case of HA-B with an initial M_w of about 1.14 × 10^6, the value after the fifth measurement was only reduced to 90%. From these results, it was concluded that the cause of the reduction in apparent viscosity of HA solutions during viscometric measurement could be attributed to degradation of polymer chains, rather than simply to disentanglement of the characteristic network. It was also suggested that HA chains with higher molecular weight were easily degraded.

Degradation Profiles with Different Shearing Rates

The effects of the shearing rate on degradation of HA chains were observed at several stages of runs. 0.1% HA-A and HA-B in 0.2*M* NaCl were used as the samples. Figure 2 shows degradation curves at various shearing rate, expressed as percentage reductions from the initial M_w values. HA-A was degraded in all runs at any shearing rate, even under the low rate such as 0.5 rpm. The degradation profiles were divided into two groups; at the relatively low rates of 0.5 and 1 rpm, and at higher rates of 5 rpm and above. With the former, M_w reduced slowly during the first hour, but after that decreased rapidly so that by 4 h the M_w became smaller than the corresponding values for samples sheared more vigorously. On the other hand, in the latter case M_w reduced rapidly during the first hour but after a long period, the decrease of M_w was slowed down. In contrast, HA-B showed little change of M_w at both 0.5 and 100 rpm [Fig. 2(b)], and the degree of reduction

Table I Changes in Weight-Average Molecular Weights of HA Samples During Viscometric Measurement

	Molecular Weight ($\times10^{-6}$)	
	HA-A	HA-B
Initial	2.57	1.14
After first measurement	2.34	1.13
After second measurement	2.23	1.11
After third measurement	2.11	1.05
After fourth measurement	2.05	1.05
After fifth measurement	1.83	1.03

Samples loaded were at the concentration of 0.1% in 0.2 *M* NaCl.

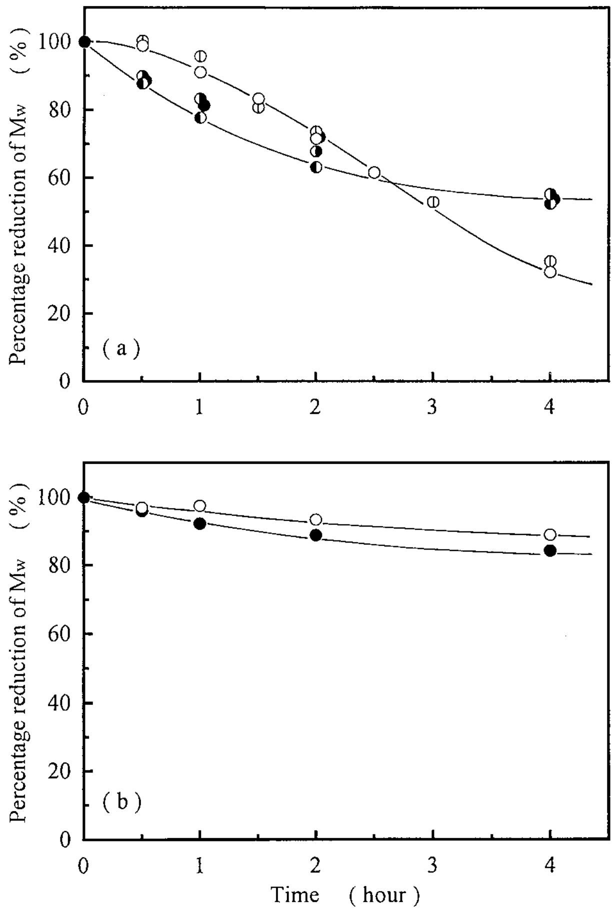

Figure 2 Degradation profiles with various shearing rates. Load was given at 37°C. (a) 0.1% HA-A in $0.2M$ NaCl; (b) 0.1% HA-B in $0.2M$ NaCl: (○) 0.5 rpm; (⦶) 1 rpm; (◑) 5 rpm; (◐) 20 rpm; (●) 100 rpm.

was only about 10% within the entire period of the experiment.

From the results indicated in Table I and Figures 1 and 2, it was concluded that higher molecular weight HA chains were easily degraded. The tendency that with mechanically applied shearing energy into the polymer molecular bonds the chain scission easily occurred in the longer polymer was like that obtained for other polymers. Porter and Johnson[7,8] and Bestul,[9,10] using polyisobutenes with various M_w, and Saita and Matumura,[12] using sodium polyacrylate, referred to that the values of initial molecular weight determined the rate of degradation. In their reports rupture of chemical bonds was considered to be induced through stretching forces on the network of polymer chains. With an increase in the molecular weight, the greater the chance of localizing mechanical shearing energy applied to the enmeshed molecules causing breaks. A similar picture could be assumed for our HA case.

Differences in Scission Behavior Depending on the Shearing Rate

Changes in RI and light scattering (LS) chromatograms with shearing rate at 0.5, 20, and 100 rpm are illustrated in Figure 3. In the case of 0.5 rpm, the peaks in the RI chromatograms of 4 h shifted obviously to a longer retention time and the heights of the LS signal became progressively smaller with time. Namely, where the amounts of shorter chains increased rapidly between 2–4 h, concurrently longer chains decreased. On the other hand, in the cases of 20 and 100 rpm, the peaks in RI chromatograms showed a little shift and the amounts of shorter chains increased, but the elution pattern for longer chains scarcely changed. The values of number average molecular weight (M_n), polydispersity (M_w/M_n), and average number of chain scission (S) are listed in Table II. S was calculated by the equation of $S = (M_{n0}/M_{nt}) - 1$, where M_{n0} and M_{nt} represent the number average molecular weights of the initial and degraded HA-A, respectively. Comparing the 0.5 and 20 rpm cases, the values of S after 4 h were almost the same, whereas the M_w/M_n were different. That of 0.5 rpm at 4 h was smaller than at 2 h. In contrast, it increased with shearing rate at 20 rpm. In polymer degradation three types of scission behavior are considered possible: central, random, and terminal. Analyses of the kinetics of mechanochemical degradation of dilute polymer solutions have generally indicated that bond ruptures occur mainly within the central regions of molecules.[6] Thus, with straight polymer molecules the distribution of applied energy will be parabolic with the peak at the center and where the stretching stress exceeds the critical value, this should be location of the breaking points. However, in practice, the picture is complicated by the fact that the molecules may not be straight but rather partially extended forms of random coils with some entanglements. Owing to the presence of a molecular meshwork, mechanical energy will not be focused exactly in the centers of single polymer chains but in the central region between two that are entangled, so that random scission can occur. But with both central and random scission, the polydispersity would not be expected to fluctuate a great deal even under drastic degradation. From the change of molecular weight and

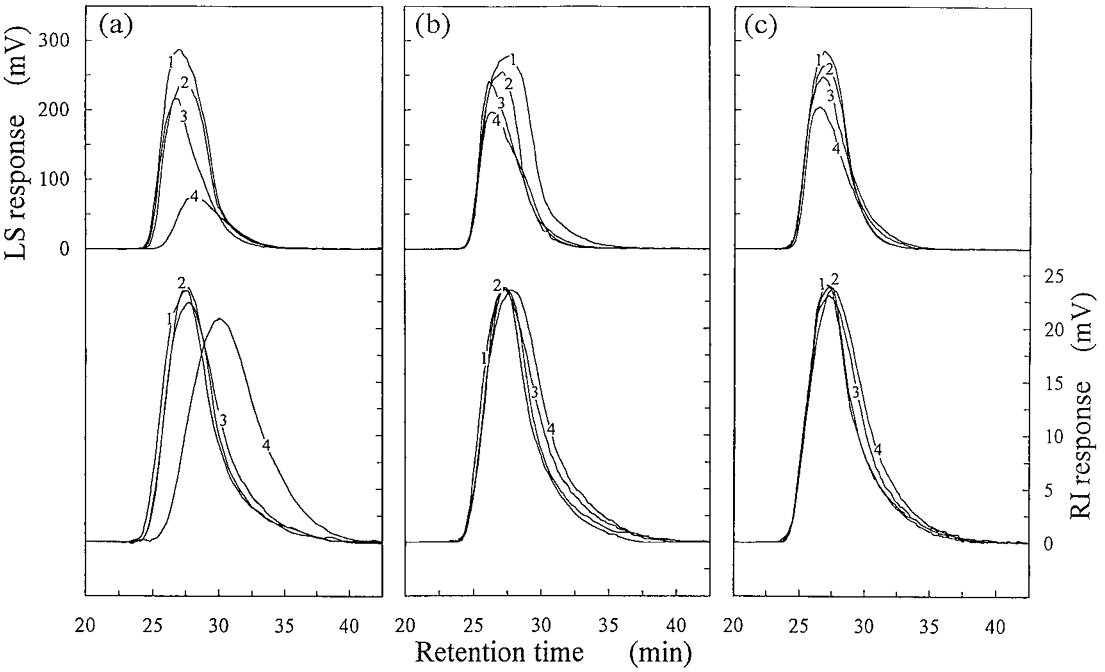

Figure 3 Changes in RI and LS chromatograms with the shearing stress. (a) 0.5 rpm; (b) 20 rpm; (c) 100 rpm: 1, initial; 2, after 1 h; 3, after 2 h; 4, after 4 h.

polydispersity, it was therefore predicted that the scission behavior in our case was terminal. Moreover, the lengths of the severed termini could be inferred to differ considerably. With degradation by terminal scission the lengths of the severed segments determine the rate of M_w decrease.[15] So it was considered that broken ends with the shearing rate of 0.5 rpm were relatively longer than that with 20 rpm. Assuming the average length of the degraded termini to be about one-fifth of the original molecules in the case of 0.5 rpm, four bonds broken per chain would accomplish the observed shift in distribution to a relatively homogeneous lower molecular weight. At 20 rpm, the severed termini would be short enough to increase the polydispersity with large amounts of long chains remaining, as evidenced by the lack of variation in the first pattern of RI chromatograms in Figure 3(b). In the case of 100 rpm, the reduction of M_w was nearly equal to that at 20 rpm, despite a much lower S value. Furthermore, the fluctuation in polydispersity was small, so as with general polymer degradation by mechanically applied shearing energy, central scission might have occurred. Because of the pseudoplastic properties of HA solutions, it was predicted that the meshwork would disentangle and individual flexible chains might be centrally ruptured by applied energy focused at their centers under such vigorous shearing conditions. Differences in the scission behavior depending on the shearing rate

Table II Molecular Weights and Polydispersities of Degraded HA-A

rpm	Time (h)	M_w ($\times 10^{-6}$)	M_n ($\times 10^{-6}$)	M_w/M_n	S
0.5	0	2.58	2.07	1.24	0.00
	0.5	2.55	2.02	1.26	0.03
	1	2.35	1.49	1.58	0.39
	2	1.72	0.66	2.60	2.14
	4	0.83	0.43	1.91	3.79
20	0	2.57	2.05	1.25	0.00
	0.5	2.31	1.62	1.43	0.27
	1	2.14	1.29	1.65	0.59
	2	1.62	0.66	2.45	2.12
	4	1.35	0.39	3.46	4.27
100	0	2.63	1.96	1.34	0.00
	0.5	2.33	1.51	1.54	0.30
	1	2.14	1.43	1.50	0.37
	2	1.90	1.14	1.67	0.72
	4	1.27	0.55	2.31	2.56

Samples were loaded at the concentration of 0.1% in 0.2 *M* NaCl.

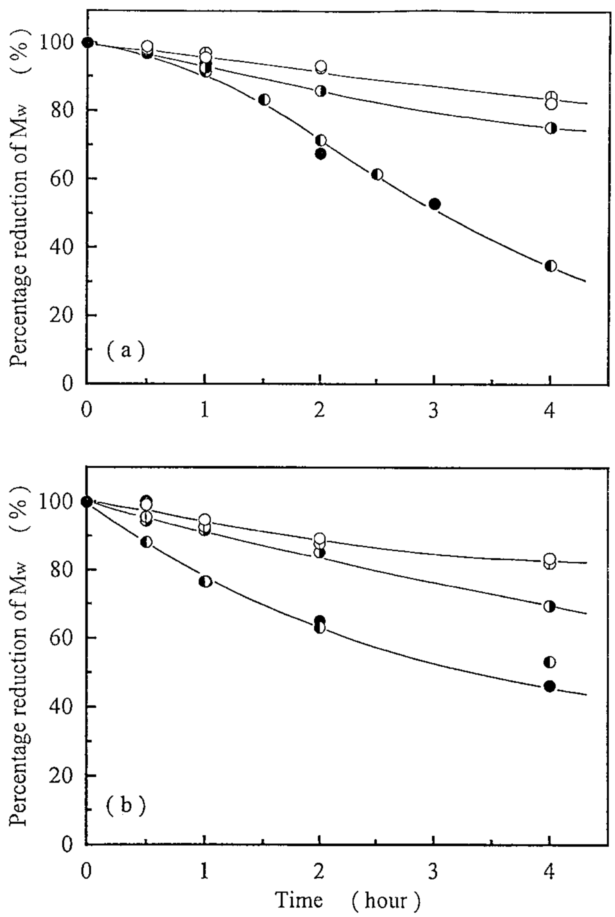

Figure 4 Effect of the NaCl concentration on degradation of HA-A in a 0.1% solution at shearing rates of (a) 0.5 rpm and (b) 20 rpm. NaCl concentrations: (○) 0.001*M*; (⦶) 0.002*M*; (◑) 0.005*M*; (◐) 0.2*M*; (●) 1*M*.

could be explained by changes in viscoelasticity reflecting molecular fluidity.

Effect of Salt Concentration

As HA is a polyelectrolyte, it dissociates in water, forming polyions. However, by adding electrolytes to the solution the electrostatic repulsion of the charged side groups along the polymer molecules will be suppressed and the chains will shrink from expanded to compact coils. Such change in the molecular size may result in alteration of the interaction among HA molecules. Therefore, effects of salt concentration on the degradation rate of HA were investigated using HA-A. We selected two shear rates, 0.5 and 20 rpm, and samples were loaded in various concentrations of NaCl, ranging from 0.001 to 1*M*. As shown in Figure 4, M_w decreased more slowly with decrease in the NaCl concentration, and the tendency was the same regardless of the shearing rate. This is contrary to the general finding that polyelectrolytes in polar solution are more easily degraded in low ionic strength because their polymer chains expand more and the chance that molecules make contact with each other increases. This difference was considered due to the viscoelasticity of the HA solution attendant on its network forming properties. By decreasing the NaCl concentration the number and density of the intermolecular entanglements would have increased because of the expansion of molecular coils. In the thick HA solution movement the individual molecule was restrained by the network formation so that substantial solid-like weak gel was displayed. As a result, the mechanical energy might not have become focused on restricted chemical bonds of the polymer main chains but rather would have been absorbed by the whole network. The energy might, therefore, not have been sufficient for effective chain scission.

Effect of HA Concentration

Also, changing the HA concentration will transform the network structure by affecting particle interactions. With increasing polymer concentration chain coils contract to a smaller size. Varying the HA-A concentration from 0.05 to 0.4% under the same 0.2*M* NaCl ionic conditions demonstrated the degradation rate to decrease with increase in the polymer concentration at both shearing rates, with the exception of 0.05% (Fig. 5). It is generally considered that the rate of polymer degradation is inversely related to the concentration.[5,11,12] The exceptional observation of 0.05% might be due to the coarseness of the network. When applying a shearing stress to the polymer, localization of energy to the chemical bonds would be expected to occur through secondary intermolecular connections such as molecular entanglements.[9] In so dilute HA solution, however, points to concentrate the stress might be scarce enough for dissipation of the applied energy by slippage of chains relative to one another. Theoretically, the number of entanglements is inversely proportional to the average molecular weight of the polymer segments between two entanglements (M_e). M_e is highly dependent upon the polymer concentration and is reported to scale exponentially.[2] According to the data offered by Onuki et al. using HA samples of various M_w from 0.02×10^6 to 2.13×10^6, the relationship was calculated as M_e

$= c^{-10.5+1.25\log M_w}$ at 37°C, where c was the HA concentration in g/mL.[16] In their experiments 1.5 mM phosphate buffer with 0.85% NaCl (pH 7.6) was used as the solvent, and this can be tentatively regarded as providing the same condition as ours with 0.2M NaCl. Adopting this expression for HA polymers with M_w of 2.57×10^6, it was calculated that one intermolecular entanglement existed per approximately 60 chains in the 0.05% solution while the figure was about one in 10 chains with 0.1%. Thus, the mesh might be considerably coarse with a concentration of 0.05%.

CONCLUSIONS

For polymer molecules of sufficient length the intermolecular bond energies are large enough to exceed the chemical bond energy of the main chain so that application of a shearing stress results in decrease in their molecular weight. The potential for degradation will be larger for polymers forming entanglement structures, which store applied energy in the secondary molecular bonds. High molecular weight HA chains interact with each other and form a temporary network structure even in a relatively dilute solution.[1,3] Our experimental findings suggest that the intermolecular bond energies in this characteristic network were large enough for scission of main chains under such conditions as encountered with routine viscometric measurement using a cone-plate rotational viscometer.

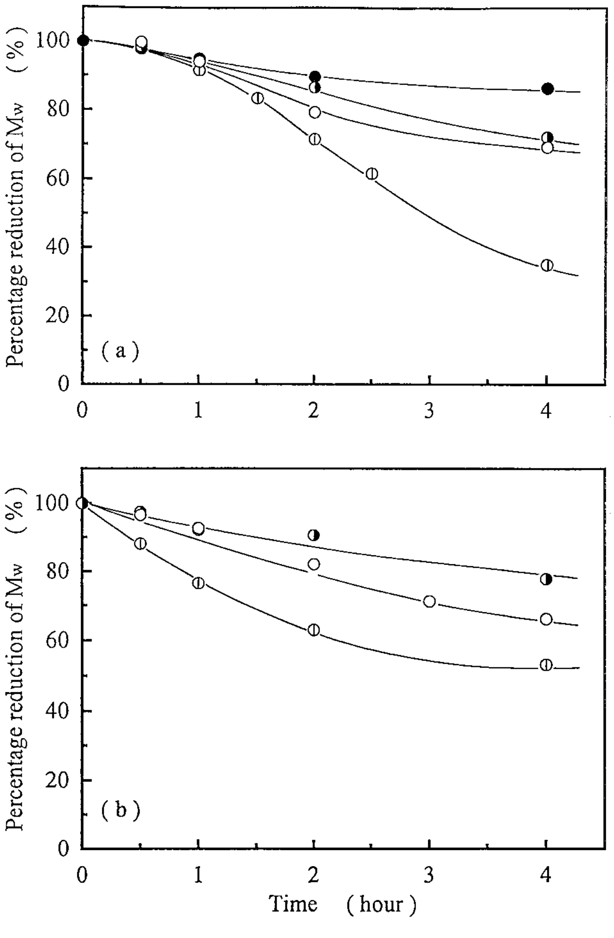

Figure 5 Effect of the polymer concentration on degradation of HA-A in a 0.2M NaCl solution at shearing rates of (a) 0.5 rpm and (b) 20 rpm. HA-A concentrations: (○) 0.05%; (⦶) 0.1%; (◑) 0.2%; (●) 0.4%.

Table III Changes in Weight-Average Molecular Weights of HA-A Solution in the Rotational Viscometer and in Test Tubes

	Reduction of M_w (%)			
	in the Viscometer with			in Test Tubes
	0 rpm	0.5 rpm	20 rpm	
Time (h)				
0	100.0	100.0	100.0	100.0
0.5	98.0	98.8	89.9	96.9
1	96.6	91.1	83.3	98.0
2	86.1	71.5	63.1	94.5
4	74.2	32.2	52.5	93.6

It must also be pointed that some decrease of M_w was noted with HA-A samples left in the cup of viscometer even without shearing (Table III). The decrease was comparatively smaller than that with shearing stress. On the other hand, with the samples in test tubes, made of glass, little decrease of M_w was evident at the same temperature of 37.0°C. Therefore, additional causes of HA chain scission other than applied shearing stress or heating could be supposed. The main difference between the two situations, in the viscometer and in test tubes, was the material with which the sample solutions contact. With regard to the degradation of HA, there are many reports concerning with the system of hydroxyl radicals catalyzed by metal ions.[17–19] Because the cup and cone of the viscometer used were made of stainless steel, effects of the metal could be suspected. In the present work we did not focus on this aspect, which will be studied in detail in the future.

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REFERENCES

1. J. E. Scott, C. Cummings, A. Brass, and Y. Chen, *Biochem. J.,* **274,** 699 (1991).
2. S. C. de Smedt, P. Dekeyser, V. Ribitsch, A. Lauwers, and J. Demeester, *Biorheology,* **30,** 31 (1993).
3. T. Yanaki and T. Yamaguchi, *Biopolymers,* **30,** 415 (1990).
4. Y. Kobayashi, A. Okamoto, and K. Nishinari, *Biorheology,* **31,** 235 (1994).
5. A. Nakano and Y. Minoura, *J. Appl. Polym. Sci.,* **21,** 2877 (1977).
6. R. E. Harrington and B. H. Zimm, *J. Phys. Chem.,* **69,** 161 (1965).
7. R. S. Porter and J. F. Johnson, *J. Phys. Chem.,* **63,** 202 (1959).
8. R. S. Porter and J. F. Johnson, *J. Appl. Physics,* **35,** 3149 (1964).
9. A. B. Bestul, *J. Chem. Phys.,* **24,** 1196 (1956).
10. A. B. Bestul, *J. Phys. Chem.,* **61,** 418 (1957).
11. T. Saita, *Jpn. J. Appl. Physics,* **19,** 2501 (1980).
12. T. Saita and O. Matumura, *Jpn. J. Appl. Physics,* **22,** 1310 (1983).
13. M. A. K. Mostafa, *J. Polym. Sci.,* **33,** 311 (1958).
14. R. S. Porter, M. J. R. Cantow, and J. F. Johnson, *J. Appl. Polym. Sci.,* **11,** 335 (1967).
15. A. Ikekawa, *Funtai Kogaku Kaishi,* **30,** 155 (1993).
16. Y. Onuki, personal communication, 1994.
17. M. J. Harris, A. Herp, and W. Pigman, *J. Am. Chem. Soc.,* **94,** 7570 (1972).
18. G. Matsumura and W. Pigman, *Arch. Biochem. Biophys.,* **110,** 526 (1965).
19. S. F. Wong, B. Halliwell, R. Richmond, and W. R. Skowroneck, *J. Inorg. Biochem.,* **14,** 127 (1981).