ELSEVIER

0144-8617(95)00046-1

Carbohydrate Polymers 27 (1995) 197–203
Elsevier Science Limited.
Printed in Great Britain.
0144-8617/95/$09.50+.00

The effect of link protein on the rheological properties of solutions of proteoglycan–hyaluronic acid aggregates

Mitsugu Tomioka* & Go Matsumura

Department of Physical Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa, Tokyo 142, Japan

(Received 1 September 1993; accepted 10 April 1995)

Proteoglycan monomers, prepared from bovine nasal cartilage, were reassembled with a commercial hyaluronic acid, with or without link protein. The effects of link protein on the rheological properties of proteoglycan aggregates were studied over the concentration range 0.1–2.0 g/100 ml. Aggregate formation of proteoglycan monomer with hyaluronic acid increased the dependence on shear rate and shifted the Newtonian region to lower shear rates, compared to that observed with a proteoglycan monomer solution at the same concentration. The dynamic viscoelasticity also increased. In the presence of the link protein there is no Newtonian flow region over the shear rate ranges at a concentration of 1 g/100 ml or above and the value of the loss tangent remained low over the angular frequencies examined. Link protein strengthens the intermolecular interactions increasing the relaxation time of the aggregate solution. It increases the co-operativity of the binding of the proteoglycan monomers to the hyaluronic acid chain allowing a dynamic network structure to be developed in solution. The rheology is strongly dependent on the weight ratio of the proteoglycan to hyaluronic acid.

INTRODUCTION

Proteoglycans are major structural components of cartilage extracellular matrix and contribute significantly to the stiffness of cartilage, its ability to immobilize water, and interactions between cartilage cells and the matrix (Muir, 1980). Proteoglycans exist as monomers and aggregates. Proteoglycan monomers consist of chondroitin sulfate and keratan sulfate chains covalently bound to a core protein. Monomers have approximately 90 chondroitin sulfate chains/monomer, 50 keratan sulfate chains/monomer and a molecular weight of approximately 2.6×10^6 (Hascall & Sadjera, 1970; Pasternack *et al.*, 1974).

In native cartilage, most of the proteoglycan monomers exist in the form of proteoglycan aggregates formed by approximately 100 proteoglycan monomers non-covalently bound to a hyaluronic acid molecule together with a link protein. The link proteins have a molecular weight of about 45,000. They associate both with the hyaluronic acid binding region of the proteoglycan core and with the hyaluronic acid chain, and stabilize the reversible, non-covalent binding between proteoglycan monomers and hyaluronic acid. Aggregates can form in the absence of link protein, but those formed with link protein are more stable and have a larger sedimentation coefficient (Hascall & Sadjera, 1969; Hardingham & Muir, 1974; Hascall & Heinegard, 1974; Rosenberg, 1975; Hascall & Kimura, 1982).

Gel chromatography studies (data not published) have shown that aggregates formed in the presence of link protein have more proteoglycan monomer molecules on a hyaluronic acid chain, in comparison to those formed without link protein. The electron microscopic measurements also show that link proteins shorten the distance between proteoglycan monomers along the hyaluronic acid chain and increase the regularity of the distance between the proteoglycan monomers (Rosenberg *et al.*, 1975; Buckwalter *et al.*, 1984). Mow *et al.* studied the rheological properties of bovine cartilage proteoglycan monomer and aggregate at concentrations similar to those found *in situ* (Mow *et al.*, 1984). They reported substantial differences in the rheological properties between the native aggregate and monomer solutions. However, little has been discovered about the effect of link protein on the physico-chemical properties of the aggregates.

*Author to whom correspondence should be addressed.

In this study, proteoglycan monomer and link protein isolated from bovine nasal cartilage were reassembled with commercial hyaluronic acid under various aggregate conditions. We attempt to elucidate the effects of link protein and the weight ratio of proteoglycan monomer to hyaluronic acid (PG/HA) on the rheological properties of PG aggregates measured under physiological conditions.

MATERIALS AND METHODS

Chemicals

All reagents were analytical grade except for guanidine hydrochloride (Gdn-HCl) and cesium chloride (CsCl). Gdn-HCl (specially prepared reagent) was obtained from Nakarai Chemical, Ltd, Japan. CsCl (density gradient grade) was from Wako Pure Chemical Industries, Ltd, Japan. HA from rooster comb (SPH, Molecular weight (MW) = 1.2×10^6) was supplied by Seikagaku Kogyo Co., Ltd, Japan.

Isolation of PG and LP

To remove non-aggregatable PGs, diced bovine nasal cartilage was washed by slowly stirring for 24 h at 4°C in 5 volumes of 0.1 M NaCl/0.05 M sodium acetate (pH 5.8) containing proteinase inhibitors. Extracts were filtered and centrifuged at 9000 r.p.m. for 30 min at 4°C. Supernatants were recovered, then dialysed against 9 volumes of 0.05 M sodium acetate (pH 5.8) for 24 h at 4°C. Equilibrium density gradient centrifugation under associative conditions was carried out in 3.5 M CsCl/0.05 M sodium acetate (pH 5.8) at 10°C for 70 h at 124,500 g_{av}.

The gradient was divided into six equal fractions, called A1–A6. The bottom fraction (A1) was dialysed exhaustively at 4°C against water, then lyophilised.

PG and link protein were prepared as follows. The above fraction was dissolved in 4.0 M Gdn-HCl/0.05 M sodium acetate (pH 5.8) at 4°C and stirred overnight to give a final concentration of about 0.2 g/ml. Equilibrium density gradient centrifugation under dissociative conditions was carried out in 3.0 M CsCl/4.0 M Gdn-HCl/0.05 M sodium acetate (pH 5.8) at 5°C for 70 h at 124,500 g_{av}. The gradient was divided into six equal fractions, called A1D1–A1D6. PG fraction (bottom A1D1) and link protein fraction (A1D6) were dialysed against water, then lyophilised.

Reassembly of proteoglycan aggregates in the presence and absence of link protein

Dilute solutions of PG, HA and link protein (LP) were prepared separately in 4.0 M Gdn-HCl/0.05 M sodium acetate (pH 5.8). Solutions of the PG and HA were combined to give a PG/HA ratio of 100, 300 and 500 with or without LP. The final concentration of PG was approximately 0.5 g/100 ml. For LP containing aggregates, the ratio by weight of proteoglycan monomer to LP was 20. The LP content of the aggregate was approximately 4.8%. Excess amounts of LP were used to yield the maximum amount of aggregate from the mixed solution of PG and 0.3% HA. In this study, HA could bind approximately 300 times by weight of HA, thus PG/HA = 300 was an approximately equivalent aggregating condition.

After mixing, the solution was allowed to stand overnight at 4°C. Each solution was then dialysed against 9 volumes of 0.05 M sodium acetate (pH 5.8) for 24 h at 4°C and subsequently water, then lyophilised separately.

Viscosity of dilute solutions

A capillary viscometer (Cannon–Manning semimicro, No.100) was used for determination of the intrinsic viscosities of PG and its aggregates. Measurements were carried out at 37°C and in 0.1 M NaCl/0.05 M Tris–HCl (pH 7.0) at a concentration range of 0.01–0.1 g/ml. The intrinsic viscosity was estimated by extrapolating the reduced viscosities to zero concentration (Huggins, 1942).

Rheological measurements

PG or its aggregates were dissolved in 0.05 M Tris–HCl buffer, pH 7.0, containing 0.1 M NaCl, at concentrations ranging from 0.1 to 2 g/100 ml, by standing for 48 h at 4°C. The concentrated solutions were centrifuged at 3000 r.p.m. for 5 min to remove air bubbles.

The solutions were tested using a cone-plate (cone angle 0.06 rad and radius 4.0 cm) or a coaxial-cylinder type rheometer (Shimadzu RM-1, equipped with a reduction gear, RDG-1). The shear rate available ranged from 1.52×10^{-2} to 4.34×10^2 s^{-1}. All measurements were carried out at room temperature (21 ± 2°C).

Dynamic viscoelastic measurements were measured at concentrations of 1 and 2 g/100 ml. The angular frequency employed ranged from 1.18×10^{-2} to 2.35 rad/s. The rotation angle of the plate and the twist angle (torque) of the cone were recorded with an X–Y plotter. From the Lissajous figure obtained the phase angle and hence the storage modulus (G′) and the loss modulus (G″) were calculated. Loss tangent ($\tan\theta$) is calculated from

$$\tan\theta = G''/G'.$$

RESULTS AND DISCUSSION

Effect of LP on the rheological properties of proteoglycan aggregate (PG/HA = 300)

Dilute solution viscosity

Figure 1 shows the concentration dependence of the reduced viscosity for PG and its aggregates. For a PG

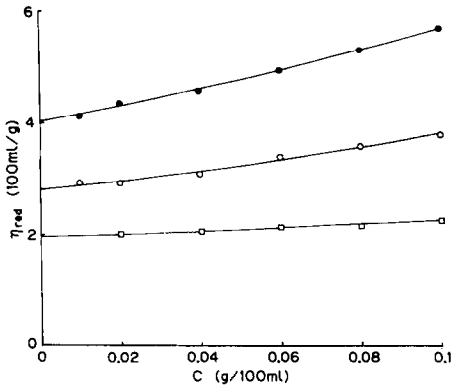


Fig. 1. Concentration dependence of reduced viscosity (η_{red}) of proteoglycan monomer and its aggregates (PG/HA = 300). Open squares show PG, open circles LP free proteoglycan aggregate; and solid circles LP containing proteoglycan aggregate.

Table 1. Intrinsic viscosities of proteoglycan monomer and its aggregates (PG/HA = 300)

Sample	Link protein	Intrinsic viscosity (100 ml/g)
Proteoglycan	–	1.97
Aggregate	–	2.82
	+	4.02

A capillary viscometer (Cannon–Manning semimicro, No. 100) was used for the determination of the intrinsic viscosity.

solution, little concentration dependence of reduced viscosity was observed. However, aggregate formation increased the reduced viscosity and the concentration dependence. As shown in Table 1 the intrinsic viscosity of the PG was increased by the formation of the aggregate. Even higher values of reduced viscosity (Fig. 1) and intrinsic viscosity (Table 1) were observed with LP containing aggregate. The number of proteoglycan monomers bound on HA chain may be increased by the presence of LP.

Concentration dependence of steady shear viscosity

As shown in Fig. 2a, PG solution behave as a Newtonian liquid at concentrations below 1 g/100 ml. However, proteoglycan aggregate solutions (Fig. 2b) showed the shear rate dependence of the specific viscosity except at the solution of lowest concentration (0.2 g/100 ml). Specific viscosity values of this aggregate were increased, compared with those of PG of similar concentration (Fig. 2a). The dependence of the specific viscosity on shear rate showed Newtonian at low shear rates. The onset of Newtonian behaviour was observed at lower shear rates as the solution concentration increased. This suggests that proteoglycan aggregate interacts to form a gel-like structure in solution.

LP containing aggregates showed an enhanced shear rate dependence of specific viscosity (Fig. 2c). In the

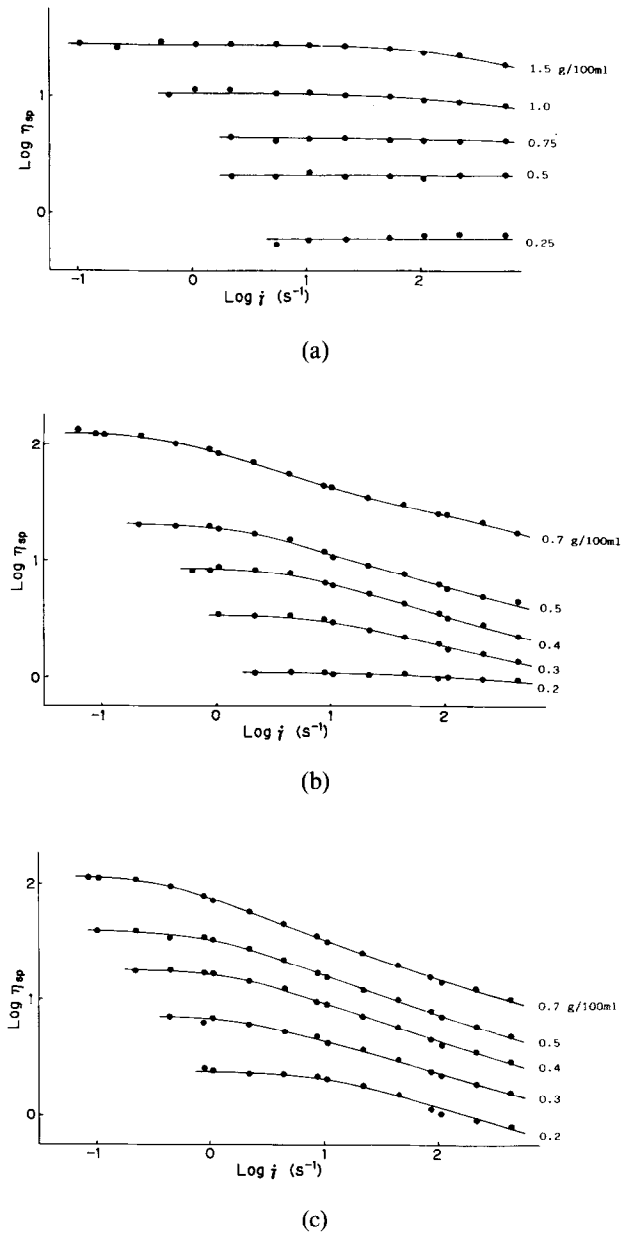


Fig. 2. Variation of specific viscosity (η_{sp}) with concentration and shear rate ($\dot{\gamma}$) for PG and its aggregates (PG/HA = 300). (a) PG; (b) LP free proteoglycan aggregate; and (c) LP containing proteoglycan aggregate.

high shear rate region, the viscosity of this aggregate was only slightly higher than that of LP free aggregate. The specific viscosity in the Newtonian region, that is, the 'zero-shear' viscosity value (Morris *et al.*, 1981), of LP containing aggregate was about twice that of LP free aggregate (Fig. 2b) and, the Newtonian flow region was shifted to lower shear rates. The presence of LP may strengthen the intermolecular interaction resulting in a more stable, gel-like structure in solution.

Figure 3 shows the shear rate dependence of the specific viscosity of solutions of the PG and its aggregates' solutions at concentrations of 1 g/100 ml. The same tendency was observed at 2 g/100 ml concentration solu-

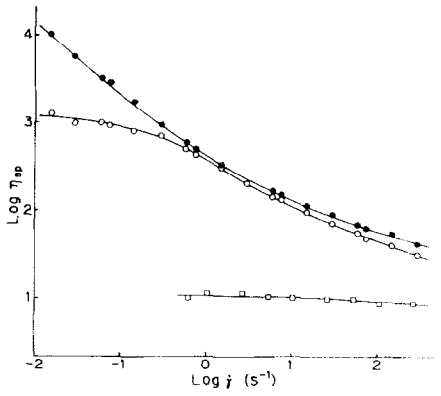


Fig. 3. Shear rate ($\dot{\gamma}$) dependence of specific viscosity (η_{sp}) for 1 g/100 ml PG and its aggregates (PG/HA = 300). Symbols as in Fig. 1.

tions. PG solutions behaved like Newtonian liquids even in these highly concentrated solutions. However, marked non-Newtonian behaviour was induced by aggregate formation. Aggregation of the PG with HA increased the 'zero-shear' viscosity (approximately 100-fold), and the solution showed Newtonian behaviour only at low shear rates, compared to that of PG monomer solutions. It seems that aggregates interact to form intermolecular linkages, either chemical or physical, of longer relaxation time. In other words, a gel-like structure develops in the aggregate solution (Flory, 1953).

The presence of LP enhanced this tendency. As shown in Fig. 3, the specific viscosity of the LP containing aggregate solution was markedly increased in the low shear rate range. No Newtonian flow region was observed over the shear rate range examined. LP may strengthen the intermolecular interactions to increase the relaxation time of a gel-forming linkage.

Dynamic viscoelasticity

Figure 4a shows the angular frequency dependence of the dynamic viscoelasticity for a PG and its aggregate solutions at 2 g/100 ml concentration. The effects of LP and its concentration on the angular frequency dependence of the loss tangent are shown in Fig. 4b.

PG solutions behaved as a low viscosity liquid with high values of the loss tangent and low values of the moduli even at concentrations of 2 g/100 ml.

Aggregate formation without the LP increased both moduli and decreased the loss tangent values. At high angular frequency, both 1 and 2 g/100 ml aggregate solutions showed low loss tangent values of approximately 0.8. The loss tangent of 1 g/100 ml solution, however, markedly increased with increasing angular frequency. In the low concentration solutions, it appeared that the lifetime of the gel-forming linkage was not strong enough to be retained at low frequencies. In other words, the relaxation time of the gel-forming linkage was rather short. For LP containing aggregate solutions, both moduli were further increased particu-

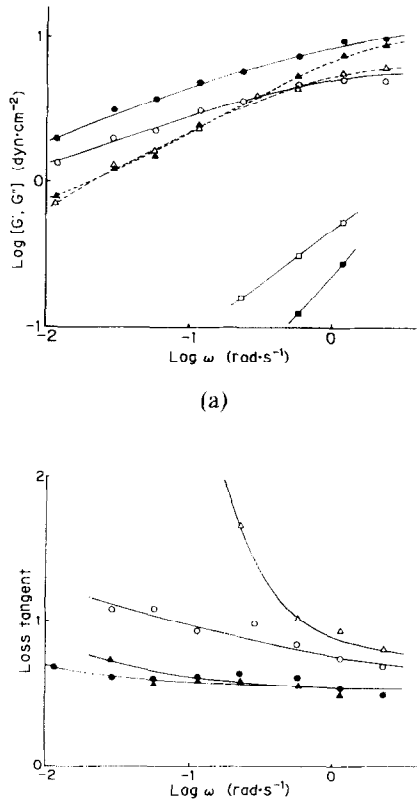


Fig. 4. (a) Angular frequency (ω) dependence of the dynamic viscoelasticity for 2 g/100 ml PG and its aggregates (PG/HA = 300). Squares show PG; triangles LP free proteoglycan aggregate; and circles LP containing proteoglycan aggregate. Filled symbols show the storage moduli (G′) and open symbols the loss moduli (G″). (b) Angular frequency (ω) dependence of loss tangent for proteoglycan aggregates (PG/HA = 300). Triangles show 1 g/100 ml solutions and circles 2 g/100 ml solutions. Open symbols show LP free proteoglycan aggregates and filled symbols LP containing proteoglycan aggregates.

larly at low angular frequencies. The value of the loss tangent remained low (approximately 0.7) even at low angular frequencies, regardless of the change in concentration. Thus, it appears that LP may increase the relaxation time of a gel-like structure in an aggregate solution (Rees *et al.*, 1982).

Effects of PG/HA ratio and LP on the rheological properties of proteoglycan aggregates

Dilute solution viscosity

Intrinsic viscosities of proteoglycan aggregates with PG/HA ratios of 100, 300 and 500 are listed in Table 2. The intrinsic viscosity of PG/HA mixtures decreased with increasing PG/HA ratio. This may be explained by an increase in the fraction of free PG that did not participate in aggregate formation. However, intrinsic viscosity values for LP containing aggregates were not

Table 2. Intrinsic viscosities of proteoglycan aggregates

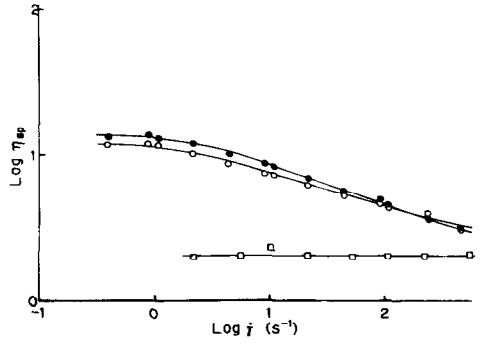
PG/HA ratio (w/w)	Link protein	Intrinsic viscosity (100 ml/g)
100	–	3.30
	+	3.85
300	–	2.82
	+	4.02
500	–	2.59
	+	3.69

A capillary viscometer (Cannon–Manning semimicro, No. 100) was used for the determination of the intrinsic viscosity.

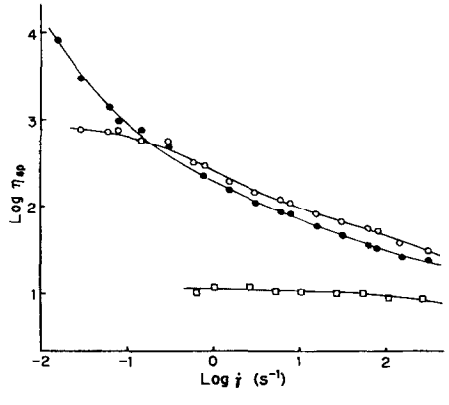
affected significantly by changes in the PG/HA ratio. PG may bind to HA chains effectively and co-operatively in the presence of LP.

Rheological behaviour

Figures 5a and b show the shear rate dependence of the specific viscosity, for 0.5 and 1 g/100 ml PG and its aggregate solutions of PG/HA ratio of 500, with or without LP respectively. The PG/HA ratio of 500 is



(a)

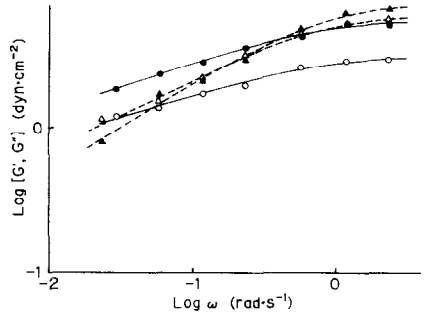


(b)

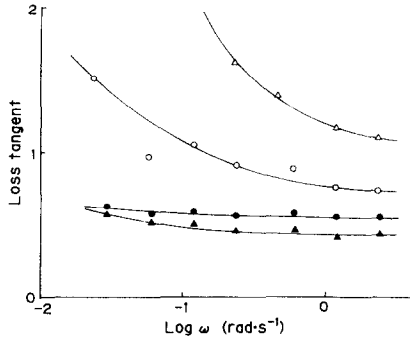
Fig. 5. Shear rate ($\dot{\gamma}$) dependence of specific viscosity (η_{sp}) for PG and its aggregates (PG/HA = 500). (a) 0.5 and (b) 1 g/100 ml solutions. Symbols as in Fig. 1.

the excess condition of PG to HA. The shear rate dependences of these aggregate solutions were similar to those of the aggregate of PG/HA ratio of 300 at the same concentration (Fig. 3). Aggregate formation increased the zero-shear viscosity (approximately 6 and 100 times, for 0.5 and 1 g/100 ml solution, respectively) and shifted the Newtonian flow region to lower shear rates, compared with those of PG solutions. For LP containing aggregate at 1 g/100 ml solution, no Newtonian flow region was observed in the shear rate range examined (Fig. 5b). The effect of LP, however, was small for this aggregate condition. The viscosity values of these LP free and containing aggregate were small over the shear rate ranges examined, compared to the aggregates of PG/HA ratio of 300 at the same solution concentration. These are also suggested by the measurement of dynamic viscoelasticity. Aggregate formation increased both moduli (Fig. 6a), compared with those of PG solution, and showed low loss tangent values (approximately 0.8–1.2) at high angular frequency (Fig. 6b). But, the loss tangent value of this LP free aggregate showed a larger concentration dependence and also a higher angular frequency dependence even at 2 g/100 ml solution, compared with those of PG/HA ratio of 300. In the presence of LP, the loss modulus gave lower values than those found for LP free aggregate solutions in the angular frequency region examined. The values of the loss tangent remained low even at low angular frequencies. However, both the moduli for proteoglycan aggregates with and without LP were lower than those with a PG/HA ratio of 300. These results suggest the presence with a unbound PG even in the presence of LP, because of the deficiency of HA to PG.

For the LP free aggregate of PG/HA ratio of 100 (the deficiency condition of PG to HA), the shear rate dependence of viscosity (Fig. 7a) was similar to that found with a PG/HA ratio of 300 at the same solution concentration (Fig. 2b). That is, aggregate formation increased the zero-shear viscosity value (approximately 10 times) and shifted the Newtonian flow region to a lower shear rate range. As shown in Fig. 7b, LP free aggregates at a concentration of 1 g/100 ml showed a lower zero-shear viscosity compared with other PG/HA ratios. This LP free aggregate showed a larger angular frequency dependence of the dynamic viscoelasticity (Fig. 8a), and higher loss tangent values at lower angular frequencies than those observed with other LP free aggregates (Fig. 8b). This may be because, under conditions of excess HA to PG, the decrease in the number of PG bound on a HA chain increases the mobility of the HA chain. However, the largest effect of the presence of LP was observed for aggregates containing excess HA, particularly in concentrated solution (Figs 7b, 8a and 8b). The physico-chemical properties were similar to other LP containing aggre-

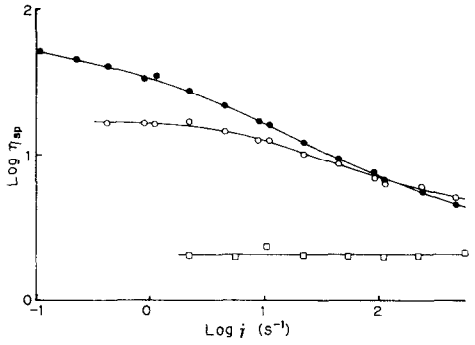


(a)

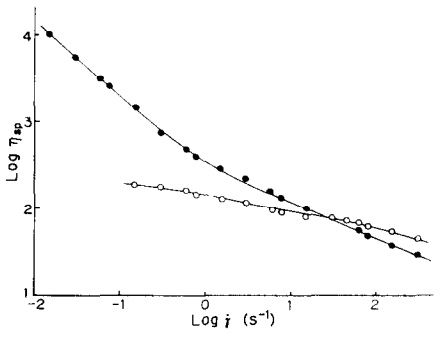


(b)

Fig. 6. (a) Angular frequency (ω) dependence of the dynamic viscoelasticity of 2 g/100 ml proteoglycan aggregates (PG/HA = 500). Triangles show LP free proteoglycan aggregates; and circles LP containing proteoglycan aggregates. Filled symbols show the storage moduli (G') and open symbols the loss moduli (G''). (b) Angular frequency (ω) dependence of loss tangent of proteoglycan aggregates (PG/HA = 500). Triangles show 1 g/100 ml; and circles 2 g/100 ml solutions. Open symbols show LP free proteoglycan aggregates; and filled symbols LP containing proteoglycan aggregates.



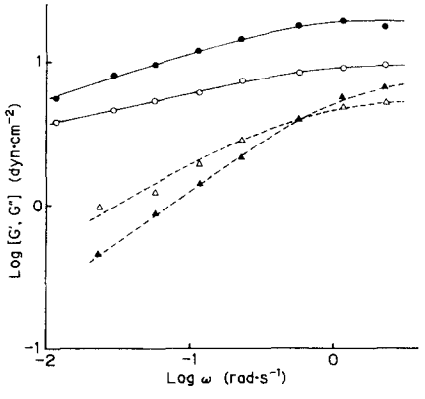
(a)



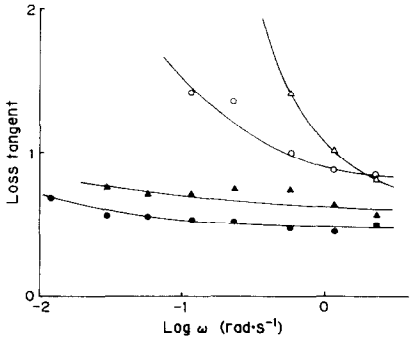
(b)

Fig. 7. Shear rate ($\dot{\gamma}$) dependence of specific viscosity (η_{sp}) for proteoglycan monomer and its aggregates (PG/HA = 100). (a) 0.5; and (b) 1 g/100 ml solutions. Symbols as in Fig. 6.

gates, that is, there was a marked shear rate dependence of viscosity and no Newtonian flow region was observed (Fig. 7b). Dynamic viscoelasticity values were further increased and angular frequency dependence of both the



(a)



(b)

Fig. 8. (a) Angular frequency (ω) dependence of the dynamic viscoelasticity of 2 g/100 ml proteoglycan aggregates (PG/HA = 100). Symbols as in Fig. 6a. (b) Angular frequency (ω) dependence of loss tangent of proteoglycan aggregates (PG/HA = 100). Symbols as in Fig. 6b.

storage and loss moduli were decreased (Fig. 8a), compared to that found with a LP free aggregate solution. The values of the loss tangent remained low over the angular frequencies examined (Fig. 8b). These suggest that LP increases co-operatively in the binding of PG on the HA chain resulting in the development of a gel-like structure in aggregate solution.

CONCLUSION

Cartilage PG solutions of concentrations at least as high as 2 g/100 ml behaved like Newtonian liquids.

Aggregate formation of PG with HA increased molecular dimensions. This resulted in interactions between macromolecules to form intermolecular linkages even in dilute solutions. This interaction developed gel-like structures with long relaxation times in the solution, particularly for concentrated solutions, and shows marked non-Newtonian behaviour and an increased dynamic viscosity.

LP should increase co-operativity in the binding of PG to the HA chain. This results in the enhancement of intermolecular interaction and elongation of relaxation time to increase the viscosity and elastic properties of the aggregate solution. This rheological property of LP containing aggregate may greatly contribute to maintaining the mechanical structure of the cartilage.

REFERENCES

Buckwalter, J.A., Rosenberg, L.C. & Tang, L.-H. (1984). *J. Biol. Chem.*, **259,** 5361.

Flory, P.J. (1953). *Principle of Polymer Chemistry.* Cornell University Press, New York.

Hardingham, T.E. & Muir, H. (1974). *Biochem. J.*, **139,** 565.

Hascall, V.C. & Heinegard, D. (1974). *J. Biol. Chem.*, **249,** 4242.

Hascall, V.C. & Kimura, J.H. (1982). *Methods in Enzymology*, Vol. 82A, edited by L.W. Lunningham and D.W. Frederiksen,. Academic Press, New York, pp. 769–800.

Hascall, V.C. & Sadjera, S.W. (1969). *J. Biol. Chem.*, **244,** 2384.

Hascall, V.C. & Sadjera, S.W. (1970). *J. Biol. Chem.*, **245,** 4920.

Huggins, M.L. (1942). *J. Am. Chem. Soc.*, **64,** 2716.

Morris, E.R., Culter, A.N., Ross-Murphy, S.B., Rees, D.A. & Price, J. (1981). *Carbohydr. Polym.*, **1,** 5.

Mow, V.C., Mak, A.F., Lai, W.M., Rosenberg, L.C. & Tang, L.-H. (1984). *J. Biomech.*, **17(5),** 325.

Muir, H. (1980). *The Joints and Synovial Fluid*, Vol 2, edited by L. Sokoloff. Academic Press, New York, pp. 27–94.

Pasternack, S.G., Veis, A & Breen, M. (1974). *J. Biol. Chem.*, **249,** 2206.

Rees, D.A., Morris, E.R., Thom, D. & Madden, J.K. (1982). *The Polysaccharides: Shapes and Interactions of Carbohydrate Chains*, Vol. 1. Academic Press Inc., New York, pp. 195–290.

Rosenberg, L.C. (1975). *Dynamics of Connective Tissue Macromolecules*, edited by P.M.C. Burleigh and A.R. Poole. Elsevier, New York, pp. 105–128.

Rosenberg, L.C., Hellmann, W. & Kleinschmidt, A.K. (1975). *J. Biol. Chem.*, **250,** 1877.