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The Role of Link-Protein in the Structure of Cartilage Proteoglycan Aggregates

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Proteoglycan fractions were prepared from pig laryngeal cartilage. The effect of link-protein on the properties of proteoglycan–hyaluronate aggregates was examined by viscometry and analytical ultracentrifugation. Aggregates containing link-protein were more stable than link-free aggregates at neutral pH, at temperatures up to 50°C and in urea (up to 4.0M). Oligosaccharides of hyaluronate were able to displace proteoglycans from link-free aggregates, but not from the link-stabilized aggregates. Both types of aggregate were observed in the ultracentrifuge, but at the concentration investigated (<2mg/ml) the link-free form was partially dissociated and the proportion aggregated varied with the pH and temperature and required more hyaluronate for saturation than did link-stabilized aggregate. The results showed that link-protein greatly strengthened the binding of proteoglycans to hyaluronate and suggest that under physiological conditions it 'locks' proteoglycans on to the hyaluronate chain.

Purified cartilage proteoglycans bind specifically to hyaluronate to form high-molecular-weight aggregates in which many proteoglycans are bound to each hyaluronate chain (Hardingham & Muir, 1972). The proteoglycans bind by a specific site at one end of the protein backbone that is largely devoid of glycosaminoglycan chains (Hascall & Heinegård, 1974*a*; Heinegård & Hascall, 1974) and has a high affinity for a decasaccharide unit of hyaluronate (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*b*). The binding can be abolished by the reduction of intramolecular disulphide bridges or by treatment with reagents that modify arginine, lysine or tryptophan residues in the protein (Hardingham *et al.*, 1976) and is very specific for hyaluronate (Christner *et al.*, 1977).

Aggregates isolated from cartilage contain, in addition to proteoglycan and hyaluronate, other 'link-protein' molecules. The link-proteins are an integral part of the aggregate structure and have been proposed to form additional bonds, possibly by bridging the proteoglycan molecule and the hyaluronate chain (Heinegård & Hascall, 1974), thereby increasing the strength of binding and giving a more stable aggregate structure. The interactions between link-proteins and proteoglycan and hyaluronate have not been characterized in detail, as the link-proteins tend to come out of solution when purified, and in the absence of this information the origin (Keiser, 1975) and specificity (Swann *et al.*, 1976) of the link-proteins have been questioned. That the link-proteins are specific components of aggregates from hyaline cartilage can be concluded from several observations. They bind to proteoglycan aggregates in concentrated salt solutions such as 3M-CsCl and are absent from those regions of a density gradient that contains other unbound proteins. From the Swarm chondrosarcoma native proteoglycan aggregates have been isolated by extraction in non-dissociative solutions, and these also contain link-protein (Faltz *et al.*, 1978). Proteolytic digestion of intact aggregates has been shown to release hyaluronate with link-protein and the hyaluronate-binding region of the proteoglycan still bound to it (Heinegård & Hascall, 1974). The protection from digestion afforded by this complex suggests a close and specific binding of these two species to the hyaluronate chain.

The link-protein fraction from bovine nasal cartilage has been shown to contain two species of molecule (mol.wt. 44000 and 48000) (Heinegård & Hascall, 1974; Baker & Caterson, 1977; Bonnet *et al.*, 1978), whereas from the Swarm rat chondrosarcoma only one type (mol.wt. 40000) was present (Oegama *et al.*, 1975). The amino acid compositions of the link-proteins and the peptides produced from them by CNBr digestion suggest that their structure was not related to that of the hyaluronate-binding region (Baker & Caterson, 1977). The similarity in composition of the two link-proteins and the ability to convert the larger form into the smaller form by limited trypsin digestion (Heinegård & Hascall, 1974) suggest that they are closely related proteins, with the larger having a peptide or glycopeptide

extension not present on the smaller form (J. R. Baker & B. Caterson, personal communication).

In the present study aggregates of proteoglycans bound to hyaluronate chains were prepared with and without link-proteins, and the effect of link-protein on the stability of the aggregate was investigated by comparing their properties by viscometry and sedimentation-velocity analysis.

Experimental

Materials

All reagents were of analytical grade except for glucosamine hydrochloride, galactosamine hydrochloride, carbazole and guanidinium chloride. The guanidinium chloride was purified with activated charcoal (Norit GSX; Hopkin and Williams, Chadwell Health, Essex, U.K.). Powdered Tyrode's medium was obtained from Difco Laboratories, East Molesey, Surrey, U.K.

Hyaluronic acid from umbilical cord was obtained from BDH Chemicals, Poole, Dorset, U.K., and a further sample from cock's comb was a gift from Professor T. C. Laurent, Uppsala, Sweden. Testicular hyaluronidase (1000 turbidity reducing units/mg) was obtained from Boehringer, Mannheim, W. Germany.

Preparation of proteoglycan fractions

Proteoglycans were extracted from pig laryngeal cartilage in 4M-guanidinium chloride and purified in CsCl density gradients as described by Hardingham & Muir (1974*a*). 6-Aminohexanoic acid (0.1M), benzamidine hydrochloride (0.005M) and disodium EDTA (0.01M) were present in the extract and associative gradients as inhibitors of proteolytic enzymes (Oegama *et al.*, 1975). The aggregated proteoglycan preparations used in the viscosity experiments were from the bottom of an associative gradient, fraction A1, and the disaggregated proteoglycans were from the bottom of a dissociative gradient, fraction A1D1 (Hardingham & Muir, 1974*a*). Link-protein was obtained from the top of a dissociative gradient (fraction A1D3).

Preparation of hyaluronate fractions

A sample of hyaluronate from cock's comb was chromatographed on a column (165cm × 1.1cm) of Sepharose 2B eluted with 0.5M-sodium acetate, pH6.8, at 6ml/h at 4°C. The eluate was analysed for uronic acid content, and three fractions eluted with K_d values of 0.0–0.2, 0.20–0.50 and 0.50–0.80 were pooled, dialysed against water and freeze-dried. [$K_d = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the sample and V_0 and V_t are the excluded and included volumes of the column respectively.]

Limiting viscosity numbers were determined on these fractions dissolved in 0.2M-NaCl and molecular weights were calculated from them as described by Cleland & Wang (1970): fraction 1, 1296ml/g (mol.wt. 671000); fraction 2, 951ml/g (mol.wt. 459300); fraction 3, 575ml/g (mol.wt. 247900).

A sample of hyaluronate (5mg) from umbilical cord was partially digested with 0.5mg of testicular hyaluronidase for 3h at 37°C in 1ml of 0.2M-sodium acetate, pH5.5. It was boiled for 3min and centrifuged at 2000*g* for 5min to remove denatured enzyme. The clear supernatant containing oligosaccharides of hyaluronate was applied to a column (120cm × 1.1cm) of Sephadex G-200 eluted with 0.5M-sodium acetate, pH6.8, at 4°C: 2ml fractions were collected, assayed for uronic acid and pooled into ten equal fractions from V_0 to V_t. The average size of the fractions was determined by rechromatographing them on the same column and comparing their elution volumes with molecular-weight calibration of the column with NaB^3H_4-reduced hyaluronate oligosaccharides (T. E. Hardingham, unpublished work). The fraction with K_d 0.52–0.59 (average mol.wt. 7500, 38 sugar residues, abbreviated $HA_{38av.}$) was used for the experiments reported here. A purified sample of decasaccharide (HA_{10}) was a gift from Dr. Halina Lis, Weizmann Institute, Rehovot, Israel.

Formation of link-free aggregates

Proteoglycan (fraction A1D1) was mixed with hyaluronate in solution under associative conditions, usually 0.5M-guanidinium chloride/0.05M-sodium acetate/1mM-EDTA, pH5.8, and left for 1h at room temperature (20–25°C) or overnight at 4°C. Link-free aggregates readily form under these conditions (Hardingham & Muir, 1972, 1974*a*,*b*).

Formation of link-stabilized aggregate

For the viscosity experiments link-stabilized aggregates were isolated from the bottom (A1) of an associative density gradient. For the sedimentation-velocity experiments link-free and link-stabilized aggregates were prepared as follows from the same A1D1 fraction and hyaluronate preparations.

Proteoglycan (fraction A1D1) was mixed with hyaluronate and link-protein fraction (fraction A1D3) under dissociative conditions in 4.0M-guanidinium chloride/0.05M-sodium acetate/1mM-EDTA, pH5.8, and then dialysed against 0.5M-guanidinium chloride/0.06-sodium acetate/1mM-EDTA, pH5.8, for 40h at 4°C. Link-protein fraction was added to fraction A1D1 in 20% higher amount than it occurred relative to proteoglycan in the dissociative density gradient by which it was prepared, as this gave a maximum proportion of aggregate.

The amount of hyaluronate added to proteoglycan

in all mixtures was based on its uronic acid content and recorded as the percentage of the total uronic acid that it accounted for (0.5–3.0%).

Viscometry

Viscosity measurements were made with an Ostwald capillary viscometer in a viscometer bath (Townson and Mercer, Croydon, Surrey, U.K.) as previously described (Hardingham & Muir, 1972). Solutions were dialysed against several changes of buffer of the required composition for 48h at 4°C and then diluted to a suitable concentration. Flow times of solutions were measured until constant, usually within 15min at 30°C. In experiments following the effect of temperature on dissociation of aggregates the solution was kept in the viscometer, and the temperature of the bath was rapidly increased in stages by using the booster heaters and measurements were taken at each stage. The temperature was then decreased by removing water and adding ice and further measurements were taken. Under these conditions the flow times were constant within 30min of attaining a new temperature (except where noted).

Analytical ultracentrifugation

Sedimentation-velocity measurements were made with an MSE Centriscan instrument in schlieren mode by using a six-place rotor (radius of mid cell 6.5cm) with 2cm-light-path cells of 1ml capacity. The relative proportions of fast-sedimenting (aggregate) and slow-sedimenting (monomer) species were determined by cutting and weighing the schlieren trace. Correction was made for radial dilution, which was less than 5% between monomer and aggregate components in those traces used for analysis. Sedimentation coefficients were determined from five timed scans of the cell by plotting $\log r$ (radial position of the peak) against $\omega^2 t$ and measuring the slope of a straight line fitted to the points by linear regression. Where aggregate peaks were noticeably skewed or polymodal the weight-average sedimentation coefficient was measured.

Analytical methods

Uronic acid was determined by an automated procedure (Heinegård, 1973) of the modified carbazole reaction (Bitter & Muir, 1962), with glucuronolactone as standard. Protein was measured by an automated modification (Heinegård, 1973) of the method of Lowry *et al.* (1951), with bovine albumin (fraction V) as standard.

The concentrations of proteoglycans and hyaluronate were routinely measured from their uronic acid contents, which were 24.8% and 42.2% respectively of the dry weights of their sodium salts. Dilutions of stock solutions of proteoglycans were carried out by weight and the final concentrations of solutions used in viscosity and sedimentation-velocity experiments were also checked by their uronic acid contents.

Results

Comparison of link-free and link-stabilized aggregates by viscometry

The characteristics of binding of proteoglycans to hyaluronate suggested that it was an equilibrium in which aggregate formation was favoured at neutral pH, at low temperature and in guanidinium chloride solutions of 0.5M or lower concentration. Aggregates were shown to dissociate reversibly at low pH (3.0) or by heating to 60°C or in 2.0M-guanidinium chloride (Hardingham & Muir, 1972, 1974*b*, 1975). The effect of various dissociating agents on proteoglycan/hyaluronate mixtures that form 'link-free' aggregates and on aggregated proteoglycan preparations (A1 fraction) that contained 'link-stabilized' aggregates were therefore compared to show if link-protein affected the characteristics of dissociation.

The results obtained with increasing concentrations of guanidinium chloride, KSCN and NaCl showed similar dissociation profiles with the two preparations (Figs. 1*a*, 1*b* and 1*c*). Both were fully dissociated in 2.0M-guanidinium chloride, pH5.8, and in 1.0M-KSCN, pH6.0, and neither was greatly dissociated in 4.0M-NaCl, pH5.8. However, in urea, pH6.0 (Fig. 1*d*), the link-free aggregate showed a progressive fall in viscosity between 1.0M- and 6.0M-urea, whereas the link-stabilized aggregate showed only a small fall in the viscosity up to 4.0M-urea and then a sharp decrease above this concentration, becoming completely dissociated in 6.0M-urea. The presence of link-protein had not increased the binding between proteoglycan and hyaluronate in the presence of the charged dissociating agents guanidinium chloride or KSCN, but did increase the binding in higher concentrations of urea.

The effect of temperature on the dissociation of the two preparations in 0.5M-guanidinium chloride, pH5.8, showed that the link-free aggregate dissociated progressively between 20 and 60°C, whereas the link-stabilized aggregate had a relatively stable viscosity up to 45°C, but when held at 55°C the viscosity fell continuously and came to a new low value after 90min (Fig. 2*a*). At 60°C it then had a viscosity comparable with that of the link-free aggregate. On cooling to 15°C its viscosity increased, but it returned to only 82% of the original value at that temperature. On recycling again to 60°C it showed a progressive fall in viscosity similar to the link-free preparation (Fig. 2*a*). The progressive fall in viscosity at 55°C (also evident at 50°C) was thus irreversible.

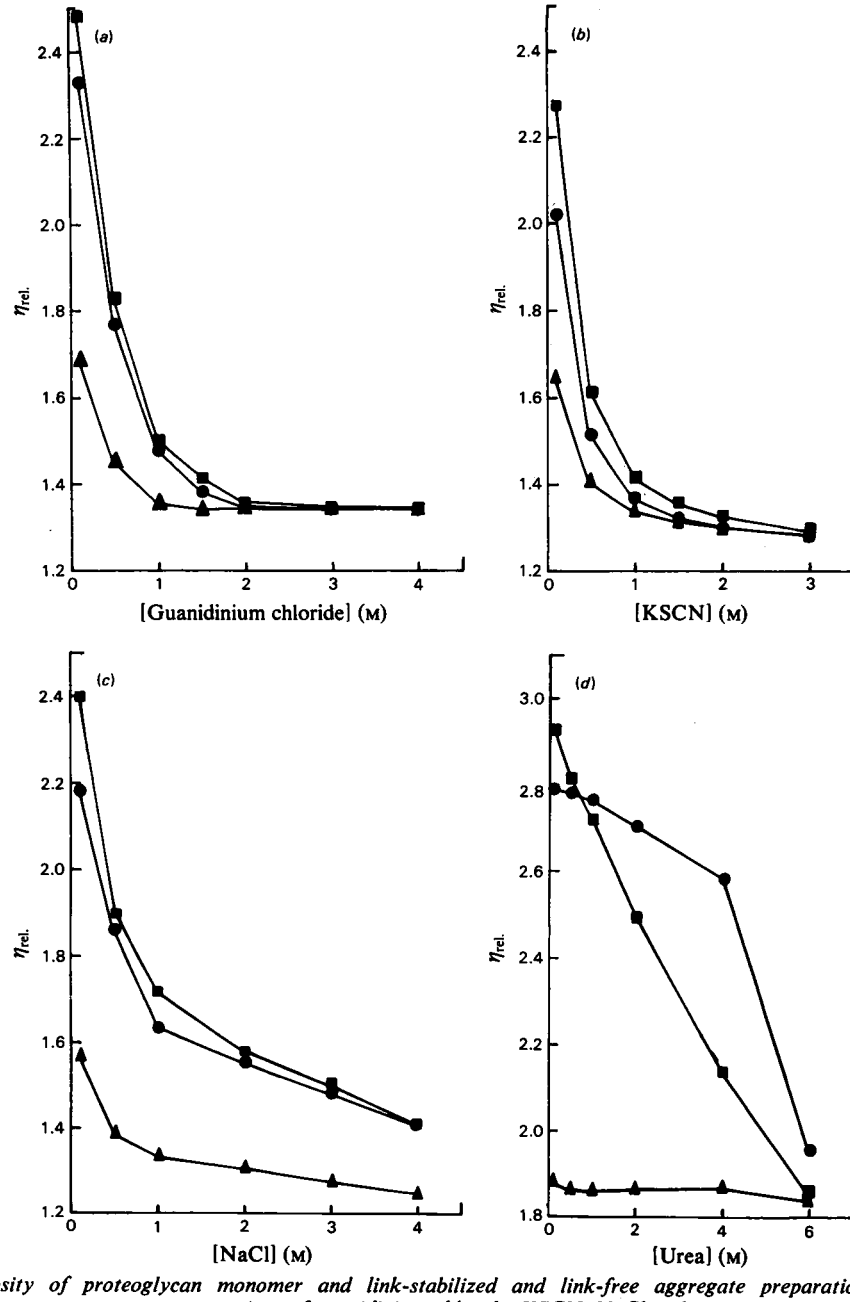

Fig. 1. *Viscosity of proteoglycan monomer and link-stabilized and link-free aggregate preparations in increasing concentrations of guanidinium chloride, KSCN, NaCl and urea*

Viscosity measurements were made on samples at: (*a*) 625μg of uronic acid/ml in guanidinium chloride/0.05M-sodium acetate, pH5.8, at 30°C; (*b*) 600μg of uronic acid/ml in KSCN/0.05M-sodium acetate, pH6.0, at 30°C; (*c*) 600μg of uronic acid/ml in NaCl/0.05M-sodium acetate, pH5.8, at 30°C; (*d*) 600μg of uronic acid/ml in urea/0.05M-sodium acetate, pH5.8, at 30°C. ●, Link-stabilized aggregate preparation (A1); ■, link-free aggregate preparation (fraction A1D1, with hyaluronate accounting for 1% of the total uronic acid); ▲, proteoglycan monomer (fraction A1D1).

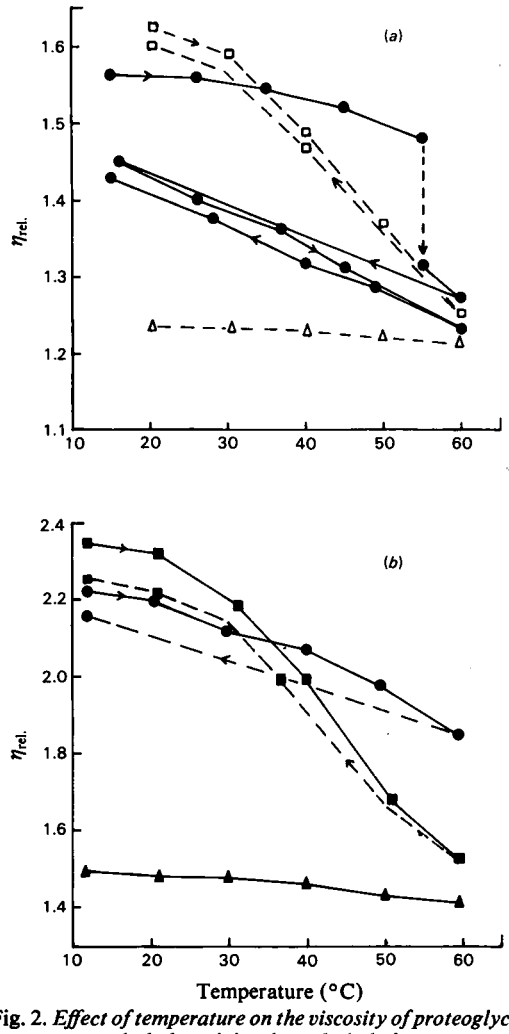

Fig. 2. *Effect of temperature on the viscosity of proteoglycan monomer and link-stabilized and link-free aggregate preparations*

Viscosity measurements were made on samples at: (*a*) 400μg of uronic acid/ml in 0.05M-guanidinium chloride/0.05M-sodium acetate, pH5.8; (*b*) 600μg of uronic acid/ml in 0.15M-NaCl buffered with 0.01M-sodium phosphate, pH7.4 (Tyrode's medium). ●, Link-stabilized aggregate preparation (fraction A1); □, ■, link-free aggregate preparation (fraction A1D1, with hyaluronate accounting for 1% of the total uronic acid); △, ▲, proteoglycan monomer (fraction A1D1).

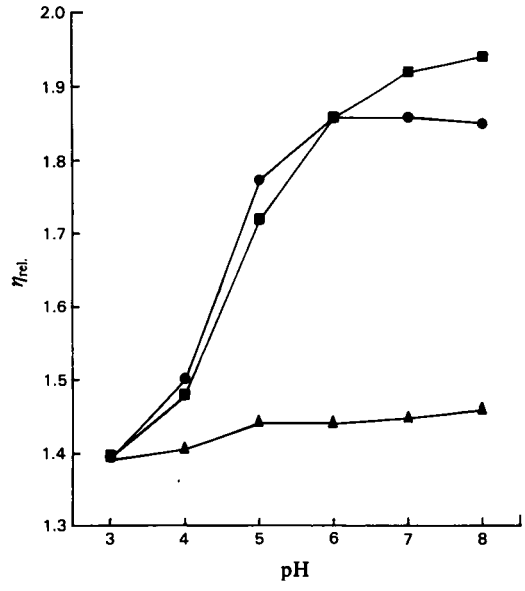

Fig. 3. *Effect of pH on the viscosity of proteoglycan monomer, link-stabilized aggregate and link-free aggregate preparations*

Viscosity measurements were made on samples at 600μg of uronic acid/ml in 0.5M-guanidinium chloride, buffered with 0.05M-sodium acetate at pH3–6 and with 0.05M-Tris/HCl at pH7–8. ●, Link-stabilized aggregate preparation (fraction A1); ■, link-free aggregate preparation (fraction A1D1, with hyaluronate accounting for 1% of the total uronic acid); ▲, proteoglycan monomer (fraction A1D1).

Link-protein increased the stability of the aggregate up to 45°C in 0.5M-guanidinium chloride, and a comparable experiment carried out in Tyrode's solution (containing 0.15M-NaCl, pH7.4) showed increased stability up to at least 60°C (Fig. 2*b*). The stabilizing effect of link-protein thus went up to and well beyond the physiological range of temperature. The hyaluronate-binding region of the proteoglycan is very resistant to thermal denaturation, i.e. in 0.5M-guanidinium chloride, pH5.8, the half-life of hyaluronate-binding activity at 80°C was 148min (Hardingham *et al.*, 1976). The appearance of link-free aggregate characteristics after heating to 60°C was thus consistent with the known properties of proteoglycan, and the irreversible loss of stability at 50°C may thus have resulted from the denaturation of link-protein. This did not appear to occur on heating the link-stabilized aggregate to 60°C in 0.15M-NaCl, pH7.4.

A comparison of the viscosities of the two preparations in 0.5M-guanidinium chloride between pH3 and pH8 showed the link-stabilized aggregate to have a broader range of stability between pH6.0 and pH8.0 (Fig. 3). Comparable experiments in Tyrode's solution also showed a fairly constant viscosity between pH5.5 and pH8.0 for link-free and link-stabilized aggregates.

On examining the effect of oligosaccharides of hyaluronate on the viscosity of link-free and link-stabilized aggregates from bovine nasal septum, Hascall & Heinegård (1974*b*) showed an important distinction between their properties. HA_{10} and larger oligosaccharides had been shown to decrease the viscosity of a solution of link-free aggregate by competing with hyaluronate for the binding site on the proteoglycan (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*b*). However, the viscosity of a solution of link-stabilized aggregate was not affected by oligosaccharides and they were thus unable to compete with binding. This was presumably because the binding of proteoglycan to hyaluronate was much stronger in the presence of link-protein. This effect with HA_{10} was confirmed with the two preparations from pig laryngeal cartilage used in this study (Fig. 4). The difference in stability of the link-stabilized and link-free aggregates was also observed with larger oligosaccharides ($HA_{38av.}$). These were potentially better competitors of binding with the link-stabilized aggregates, as they would be large enough to bind both link-protein and proteoglycan (Kimura *et al.*, 1978), but the results show that the binding in the presence of link-protein is not open to competition even by the larger oligosaccharides.

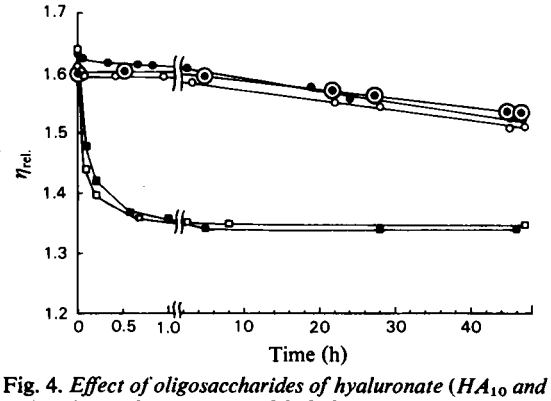

Fig. 4. *Effect of oligosaccharides of hyaluronate (HA_{10} and $HA_{38av.}$) on the viscosity of link-free and link-stabilized aggregates*

Viscosity measurements were made on proteoglycan solution in 0.5 M-guanidinium chloride/0.05 M-sodium acetate/0.001 M-disodium EDTA, pH 5.8, at 30°C. Link-stabilized aggregate (fraction A1) was at 492 μg of uronic acid/ml alone (⊙) or after the addition of HA_{10} (●) or $HA_{38av.}$ (○) accounting for 2% of the total uronic acid. Link-free aggregate (fraction A1D1, with hyaluronate accounting for 1% of the total uronic acid) was at 498 μg of uronic acid/ml after the addition of HA_{10} (■) or $HA_{38av.}$ (□) accounting for 2% of the total uronic acid.

Analysis in the centrifuge

The link-stabilized aggregate normally isolated from cartilage as a fraction (A1) at the bottom of an associative CsCl gradient was originally characterized as a species of high sedimentation coefficient (Sajdera & Hascall, 1969). However, the link-free aggregate has been at various times observed (Swann *et al.*, 1976) or not observed (Hardingham & Muir,

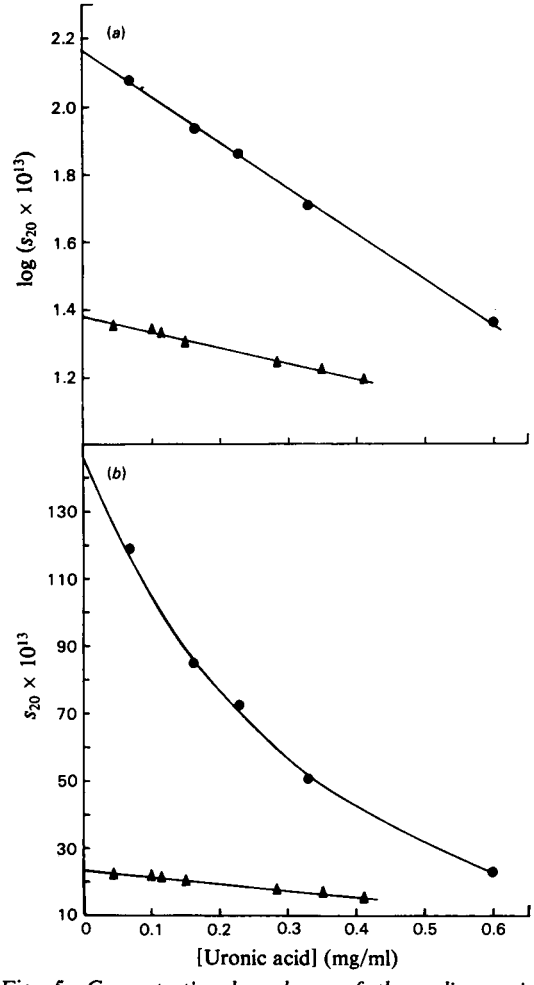

Fig. 5. *Concentration-dependence of the sedimentation coefficients of proteoglycan monomer (fraction A1D1) and link-stabilized aggregate (fraction A1) preparations in 0.5 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, at 30°C*

(*a*) log($s_{20} \times 10^{13}$) plotted against concentration of proteoglycan; (*b*) $s_{20} \times 10^{13}$ plotted against concentration of proteoglycan. ●, Link-stabilized aggregate preparation (fraction A1); ▲, proteoglycan monomer (fraction (A1D1).

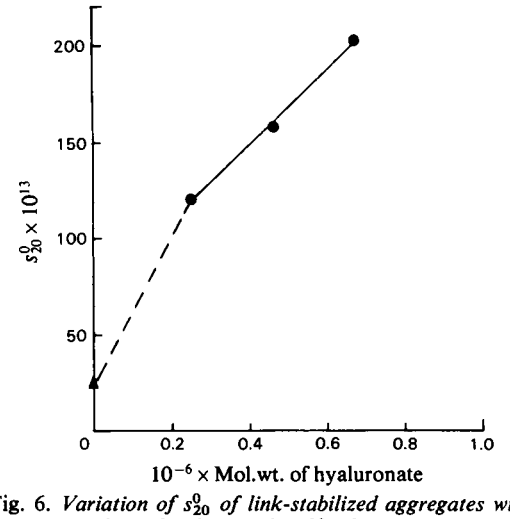

Fig. 6. *Variation of s^0_{20} of link-stabilized aggregates with the molecular weight of hyaluronate*

1972; Gregory, 1973; Oegama *et al.*, 1977) without any reasonable explanation. The behaviours of link-stabilized and link-free aggregates were thus examined in detail in an analytical ultracentrifuge.

The sedimentation coefficient of the link-stabilized aggregate A1 shows a strong concentration-dependence that is non-linear. It is shown in Fig. 5(*a*): (*a*) fitted to a straight line on a semi-logarithmic plot and

Link-stabilized aggregates were formed as described in the text from fraction A1D1 with a constant proportion of hyaluronate subfraction (accounting for 0.5% of total uronic acid) and dialysed against 0.5M-guanidinium chloride/0.05M-sodium acetate, pH5.8. s^0_{20} for the aggregate in each preparation was determined from a semi-logarithmic plot (Fig. 5*a*) of sedimentation coefficient against concentration for five dilutions from 100 to 500μg of uronic acid/ml. ▲, s^0 for proteoglycan monomer.

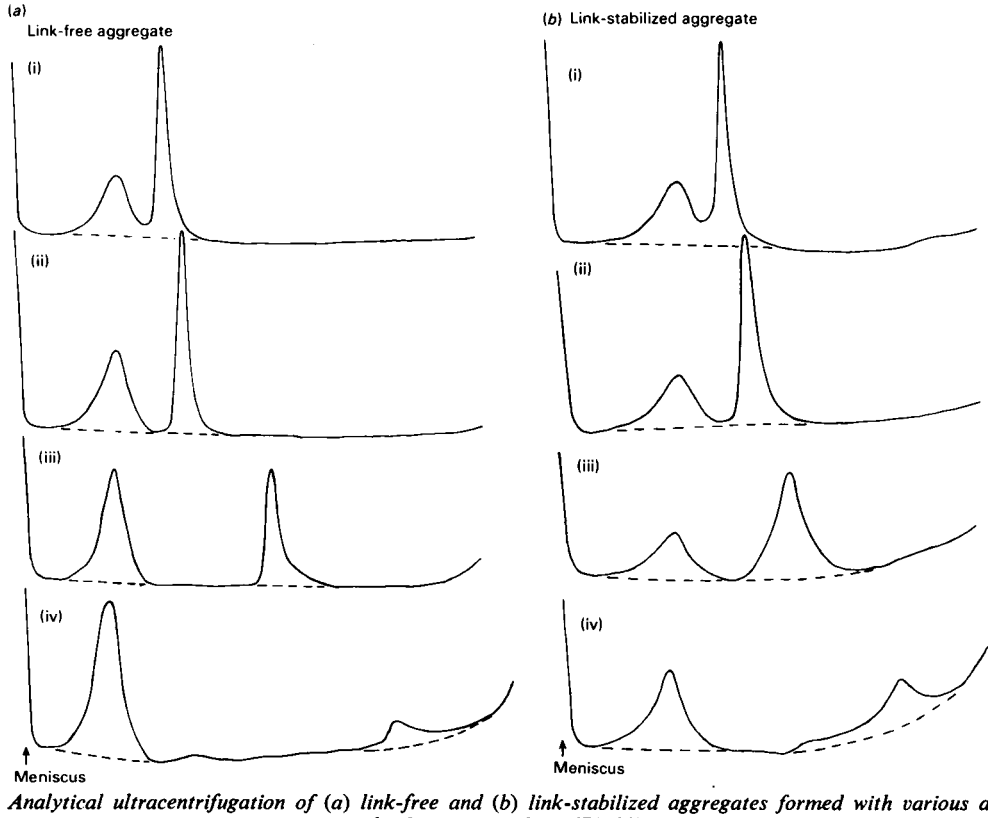

Fig. 7. *Analytical ultracentrifugation of (a) link-free and (b) link-stabilized aggregates formed with various amounts of hyaluronate (mol.wt. 671100)*

Aggregates in which the proportion of total uronic acid present in hyaluronate was (i) 3.0%, (ii) 2.0%, (iii) 1.0% and (iv) 0.5% were prepared as described in the text and centrifuged at 400μg of uronic acid/ml in 0.5M-guanidinium chloride/0.5M-sodium acetate/1mM-EDTA, pH5.8, at 20000rev./min at 20°C. Schlieren scans are shown for each sample at approximately the same time after attaining speed. The direction of sedimentation is left to right.

(*b*) plotted normally. The former was used to give a more objective assessment of s^0 by avoiding non-linear extrapolation that is necessary with normal or $1/s$-versus-c plots (Rosenberg *et al.*, 1973; Pita *et al.*, 1978). The strong concentration dependence greatly restricted the range over which useful measurements could be made to 0.2–2.0mg/ml.

The effect of the molecular weight of hyaluronate on the sedimentation coefficient of the aggregate was determined with three subfractions of hyaluronate. Link-stabilized aggregates were formed with each fraction at 0.5% (w/w) uronic acid ratio relative to proteoglycan. At this ratio the amount of hyaluronate is limiting so that each chain should be maximally substituted with proteoglycans. There was a direct correlation of the s^0 of the aggregate with the molecular weight of the hyaluronate (Fig. 6). The hyaluronate subfractions, particularly that of high molecular weight, produced sharper aggregate peaks (see Fig. 7) that were easier to detect at low concentration and were therefore an advantage in measuring the proportion of aggregates and their sedimentation coefficients. They were thus used in preparing link-free and link-stabilized aggregates for comparison by sedimentation-velocity analysis.

The results of the viscosity experiments showed that the binding of proteoglycan (PG) to hyaluronate (HA) to form link-free aggregates was a dynamic equilibrium:

$$PG + HA \rightleftharpoons PG\text{–}HA \qquad (1)$$

and provisional estimates of the binding of oligosaccharides to proteoglycans (T. E. Hardingham, unpublished work) suggested that that dissociation constant was 0.1 μM:

$$K_D = \frac{[PG][HA]}{[PG\text{–}HA]} = 0.1\,\mu M \qquad (2)$$

The concentration of proteoglycan in the sedimentation-velocity runs was 0.2–2.0mg/ml, which is 0.1–1.0 μM (proteoglycan $M_n \simeq 1.63 \times 10^6$; see Hardingham *et al.*, 1976) and is close to the dissociation constant. However, the initial experiments with link-free aggregate preparations did not show obvious equilibrium effects; the aggregate formed a fast-sedimenting component well resolved from the monomer proteoglycan (see Fig. 7*b*).

To give a controlled comparison of link-free and link-stabilized aggregates they were formed from the same proteoglycan fraction A1D1 and from the high-molecular-weight hyaluronate fraction by dialysis from 4M-guanidinium chloride with and without link-protein fraction (A1D3). The proportion of aggregate was determined in preparations made with hyaluronate comprising 0.5–3.0% of the total uronic acid (Figs. 7*a* and 7*b*). With a proteoglycan concentration of 1.6mg/ml there was evidence of partial dissociation of aggregate in the link-free preparation, as with low proportions of hyaluronate there was much less aggregate than in the link-stabilized preparation (Fig. 8). It required hyaluronate at 2.0% of the uronic acid to give a maximum amount of aggregate, compared with 1.0% in the link-stabilized preparation. This suggested that the higher concentration of hyaluronate was necessary to favour complete binding in the link-free preparation.

By using serial dilutions the s^0 values of the aggregates were determined in these preparations (Fig. 9*a*), and they were found to decrease as the proportion of hyaluronate in the preparation increased (Fig. 9*b*). This was expected, as when hyaluronate was 0.5% of the uronic acid it was calculated to be less than sufficient to bind all the proteoglycans (Hardingham & Muir, 1973) and it should therefore be saturated with them, whereas at 1.0%, 2.0% and 3.0% hyaluronate would be in excess and each hyaluronate chain would therefore have fewer proteoglycans bound to it. The link-free aggregates showed a similar trend, but the concentration-dependence of sedimentation and the s^0 values were lower (Figs. 9*a* and 9*b*). At low concentrations the proportions of link-free aggregate and its sedimentation coefficient were lower, which would correspond to fewer proteoglycans being bound on each hyaluronate chain.

To see if temperature or pressure was affecting the proportion of aggregate seen in the centrifuge,

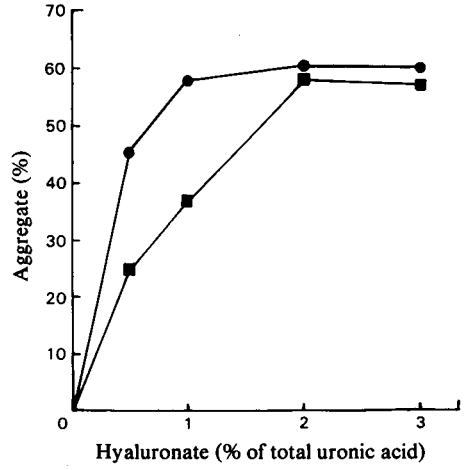

Fig. 8. *Proportion of aggregate in link-free and link-stabilized preparations with various amounts of hyaluronate*
The samples and conditions were as in Figs. 7(*a*) and 7(*b*). The proportions of link-free (■) and link-stabilized aggregate (●) were determined as described in the text.

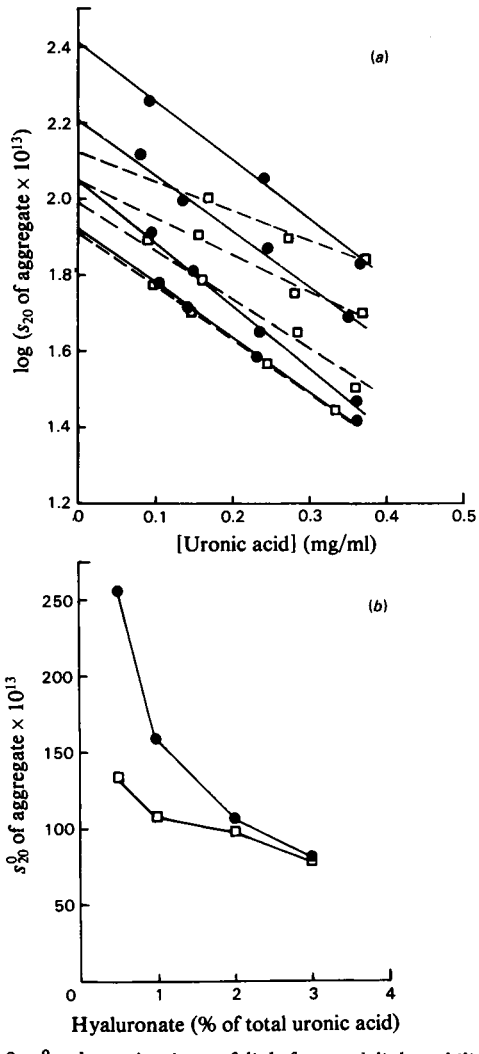

Fig. 9. s^0_{20} *determinations of link-free and link-stabilized aggregates formed with various amounts of hyaluronate*
The samples of conditions were as in Figs. 7(*a*) and 7(*b*). (*a*) Concentration-dependence of s_{20} of the link-free (□) and link-stabilized aggregate (●) formed with 0.5–3.0% hyaluronate. (*b*) Variation of s^0_{20} of the link-free (□) and link-stabilized (●) aggregates with various amounts of hyaluronate.

samples were run at different *g* values and at different temperatures (Table 1). It was immediately evident that the temperature markedly affected the proportion of link-free aggregate, but had no effect on the amount of link-stabilized aggregate. This explained some of the discrepancy between the proportion of aggregate in link-free preparations found by gel chromatography and that found in the ultracentrifuge

Table 1. *Effect of temperature and centrifugation at different speeds on the sedimentation behaviour of link-free aggregate*
The proteoglycan (2mg/ml) was in 0.5M-guanidinium chloride/0.05M-sodium acetate, pH5.8, in which hyaluronate accounted for 1% of the total uronic acid. The proportion of aggregate was determined as described in the text.

Temperature (°C)	10^{-3} × Speed of rotor (rev./min)	$10^{-3} \times g_{av.}$	Aggregate (%)
20	5	2	45
20	25	46	42
20	50	180	47
4	20	30	53
20	20	30	42
38	20	30	28

(Hardingham & Muir, 1974*a*), as the former was in 0.5M-sodium acetate, pH6.8, whereas the latter was under more dissociative conditions in 0.05M-guanidinium chloride, pH5.8, and 20°C. There was no significant variation in the proportion of aggregate at different *g* values. This suggested that there was not a large volume change on binding of proteoglycan to hyaluronate.

The apparent proportion of aggregate in a preparation decreased at concentrations above 1.5mg/ml owing to the Johnston–Ogston effect (Johnston & Ogston, 1946), as observed by Oegama *et al.* (1975) and Pita *et al.* (1978). This occurred with both link-stabilized and link-free preparations and was therefore unrelated to the presence of link-protein. This interpretation was supported by the attainment of a constant maximum proportion of aggregate at concentrations below 1.5mg/ml and by the effect being less marked when the aggregate was better separated from the monomer, such as when formed with 0.5% or 1.0% hyaluronate compared with 2% hyaluronate, or with hyaluronate of high molecular weight compared with that of low molecular weight.

Although the link-stabilized preparation showed the highest proportion of aggregate at low concentration (Fig. 10*a*), the link-free preparation showed a decreased proportion in the most dilute solutions (Fig. 10*b*). This effect appeared to be more obvious at pH5.8 than at pH7.0. A possible explanation of this was that it resulted from the dissociation of the link-free aggregate at low concentration, and the viscosity experiments (Fig. 3) showed this to be greater at lower pH. How the proportion of link-free aggregate decreased at low concentration was calculated by assuming two different values of K_D and is shown in Fig. 10. There is some general agreement of the experimental observations with the theoretical curves at the lowest concentrations where the

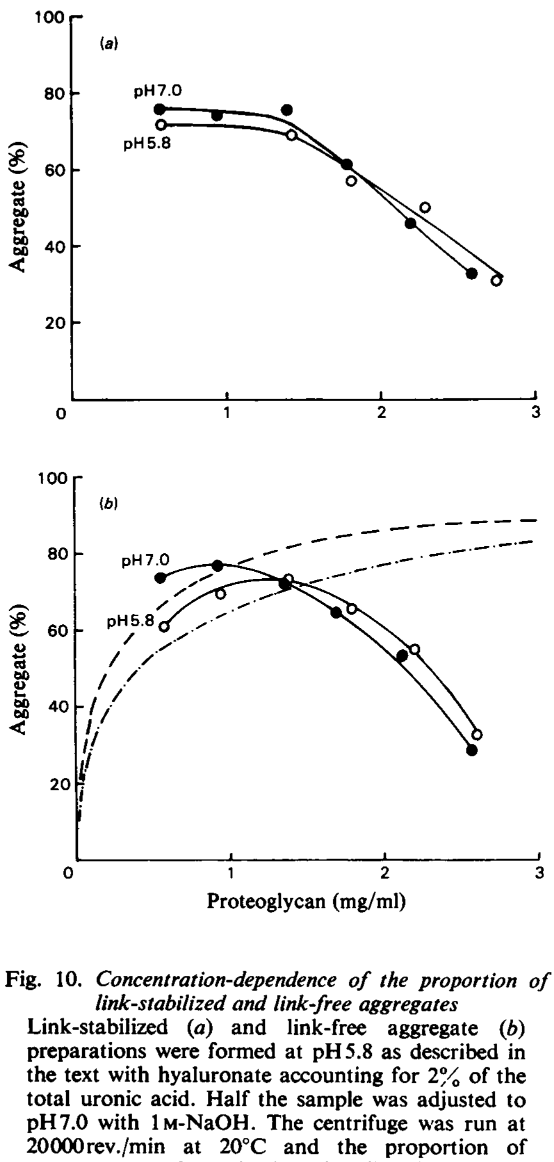

Fig. 10. *Concentration-dependence of the proportion of link-stabilized and link-free aggregates*

Link-stabilized (*a*) and link-free aggregate (*b*) preparations were formed at pH 5.8 as described in the text with hyaluronate accounting for 2% of the total uronic acid. Half the sample was adjusted to pH 7.0 with 1 M-NaOH. The centrifuge was run at 20000 rev./min at 20°C and the proportion of aggregate was determined as described in the text. The equilibrium values (*b*) were calculated from eqn. (2), assuming proteoglycan $M_n = 1.63 \times 10^6$ a molecular weight of hyaluronate bound by one proteoglycan of 10000, with a molar ratio for the actively binding proteoglycan (90% estimated for this preparation) to the available hyaluronate sites of 1:2.13. K_D was assumed to be 0.17 μM (----) and 0.36 μM (·-·-).

Johnston–Ogston effect was no longer significant. The results were not thus at variance with this interpretation, but it is difficult with the present techniques to make measurements at even lower concentrations to test the fit further.

The results of sedimentation-velocity analysis thus corroborated the conclusions from the viscosity experiments and provided further evidence that the binding of proteoglycan to hyaluronate in the link-free aggregate was made much stronger by the link-protein fraction. The proportion of aggregate seen in a sedimentation-velocity run of a link-free preparation was affected by the pH, temperature and concentration of solute and required more hyaluronate for saturation than did link-stabilized aggregate. These results may explain the lack of consistency in observing link-free aggregate in our previous less-detailed studies (Hardingham & Muir, 1972, 1974*a*).

Discussion

The results clearly showed that link-protein had a stabilizing effect on proteoglycan aggregate structure. Link-protein (LP) binds to both proteoglycan and hyaluronate (Heinegård & Hascall, 1974), but separate assessment of the strength of these interactions (eqn. 3) have not been reported:

$$PG + HA + LP = \underset{\diagdown \ \diagup \atop LP}{PG\text{——}HA} \qquad (3)$$

In the present experiments only the net binding of proteoglycan to hyaluronate is detected by viscometry or sedimentation-velocity analysis; however, some of the characteristics of link-protein interactions are evident. Where there was no apparent difference in the dissociation of the two types of aggregate, in KSCN or guanidinium chloride, dissociation must have been limited by proteoglycan binding to hyaluronate, and the binding of link-protein to proteoglycan and/or hyaluronate was therefore very weak under these conditions, but in urea and in associative solvents at neutral pH and at temperatures up to 50°C negligible dissociation occurs and both these interactions must then be considerably stronger than proteoglycan binding to hyaluronate.

With a reversible equilibrium of binding between proteoglycan and hyaluronate it may not be clear why two sedimenting species can be clearly separated in the ultracentrifuge. However, each hyaluronate chain has many proteoglycans bound to it and is likely to have 20–40 proteoglycan molecules bound to each hyaluronate chain of 5×10^5 mol.wt. It is also sedimenting through a constant concentration of monomer and its size should therefore remain constant through a sedimentation run. These considerations imply that equilibrium effects on the resolution of monomer and aggregate would only be apparent when the number of proteoglycans bound per hyaluronate chain is low.

However, this is difficult to verify experimentally, as proteoglycan polydispersity also makes resolution of monomer and aggregate difficult under these conditions.

In the present work an impure link-protein fraction A1D3 was used for most of the studies. By sodium dodecyl sulphate/polyacrylamide-gel electrophoresis this was about 90% link-protein (largely the high-molecular-weight form), but it contained in addition small amounts of proteoglycan of high protein content and trace amounts of hyaluronate and other high-molecular-weight protein components (D. Dunham & T. E. Hardingham, unpublished work). The molecular weights of the trace components (all larger than link-protein) preclude their presence in molar concentrations comparable with that of the proteoglycan and they are therefore unlikely to produce the effects observed here. Furthermore, the key results have also been repeated with a more pure link-protein prepared by enzymic digestion of aggregate (Heinegård & Hascall, 1974), and the same basic stabilization effect was observed in the ultracentrifuge (D. Dunham & T. E. Hardingham, unpublished work).

From the present results the strength of binding of link-protein to proteoglycan and to hyaluronate cannot be determined. Furthermore, these results were obtained with the high-molecular-weight link-protein and proteoglycan from pig tissue, and whether there is any functional difference between the two forms of link-protein or in link from different cartilaginous tissues or different species remains to be determined.

Precisely how proteoglycan aggregation is important to the function of cartilage is not known. It may be proposed that aggregation would effectively immobilize proteoglycans within a network of collagen fibres, thus enhancing their ability to resist compressive deformation and give better load distribution. If this is so, the role of link-protein would consolidate this advantage, but the 'locking' of proteoglycans in the aggregate may also produce a species more protected from proteolytic degradation, thus extending the useful life of proteoglycans in the matrix. On the information available it appears that aggregate formation may be an irreversible process in the tissue matrix and that turnover of proteoglycan may consequently occur only when the aggregate is degraded by a concerted proteolytic attack.

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