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Demonstration of link protein in proteoglycan aggregates from human intervertebral disc

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Proteoglycan aggregates free of non-aggregating proteoglycan have been prepared from the annuli fibrosi and nuclei pulposi of intervertebral discs of three human lumbar spines by extraction with 4M-guanidinium chloride, associative density gradient centrifugation, and chromatography on Sepharose CL-2B. The aggregate (A1-2B.V_0) was subjected to dissociative density-gradient ultracentrifugation. Three proteins of M_r 38900, 44200 and 50100 found in the fraction of low buoyant density (A1-2B.V_0-D4) reacted with antibodies to link protein from newborn human articular cartilage. After reduction with mercaptoethanol, two proteins of M_r 43000 and two of M_r 20000 and 14000 were seen. The A1-2B.V_0-D4 fraction, labelled with ^{125}I, co-eluted with both hyaluronate and a hyaluronate oligosaccharide (HA_{14}) on a Sepharose CL-2B column. HA_{10} and HA_{14} reduced the viscosity of A1 fractions; HA_4, HA_6 and HA_8 did not. HA_{14} decreased the viscosity of disc proteoglycans less than it did that of bovine cartilage proteoglycans. Thus, although a link protein was present in human intervertebral disc, it stabilized proteoglycan aggregates less well than did the link protein from bovine nasal cartilage.

Proteoglycan monomers from hyaline cartilage contain principally chondroitin sulphate and lesser amounts of keratan sulphate attached to the same core protein, the keratan sulphate predominating near the end of the molecule which terminates in a structure which binds specifically to hyaluronate (reviewed by Muir, 1980; Hardingham, 1981; Hascall, 1981). Many proteoglycan monomers combine with a single molecule of hyaluronate to form extremely large aggregates containing up to three link proteins which bind non-covalently with both hyaluronate and the hyaluronate-binding region of the core protein (Hascall & Sajdera, 1969; Baker & Caterson, 1978; Bonnet *et al.*, 1978; Tang *et al.*, 1979). The link proteins render the aggregate more resistant to dissociation by changes in pH, elevated temperature, high concentrations of urea, and hyaluronate oligosaccharides (Hardingham, 1979).

Proteoglycans of human intervertebral disc (reviewed by McDevitt, 1981) contain both aggregating and non-aggregating species (Emes & Pearce, 1975; Adams & Muir, 1976). The proteoglycan monomer contains approximately equal amounts of chondroitin 6-sulphate and keratan sulphate attached to a shared protein core (Pearce & Grimmer, 1976). Aggregate formation involves hyaluronate (Stevens *et al.*, 1979; Lyons *et al.*, 1981). Link protein has not been demonstrated previously in the intervertebral disc. In the present work the protein components of the aggregate from both annulus fibrosus and nucleus pulposus were isolated and shown to interact with both hyaluronate and proteoglycan. However, the protein was less effective than link from cartilage in stabilizing the aggregate against dissociation by hyaluronate oligosaccharides.

Abbreviations used: SDS, sodium dodecyl sulphate; HA_n, hyaluronate oligosaccharide containing n monosaccharide units.

Materials and methods

Hyaluronate

Sodium hyaluronate (nominal M_r 1.6×10^6; HA_{8000}) prepared from rooster comb (Healon) was a gift from Pharmacia AB, Uppsala, Sweden. Oligosaccharides, prepared by Sephadex G-75 chromatography of hyaluronate digested with testicular hyaluronidase (Hascall & Heinegard,

1974*a*), were identified as HA_n, where n is the number of monosaccharide units in the molecule.

Analytical methods

Hexuronate was measured by the carbazole–borosulphuric acid reaction (Bitter & Muir, 1962), hexose by the anthrone reaction (Scott & Melvin, 1953) and protein by the reaction of Lowry *et al.* (1951), using sodium glucuronate (Corn Products Refining Co., New York, NY, U.S.A.), galactose (Calbiochem-Behring, San Diego, CA, U.S.A.) and bovine serum albumin monomer standard (Miles Laboratories, Elkhart, IN, U.S.A.), respectively, as standards. SDS/polyacrylamide-gel electrophoresis with and without reduction with mercaptoethanol, using the standard conditions of Neville (1971) was done with proteins of known M_r values as standards (Electrophoresis Calibration Kit from Pharmacia). SDS was purchased from Matheson, Coleman & Bell (Norwood, OH, U.S.A.), bisacrylamide and acrylamide from Bio-Rad Laboratories (Richmond, CA, U.S.A.), and *NNN'N'*-tetramethylethylendiamine from Eastman Kodak Co. (Rochester, NY, U.S.A.). The gels were stained with Coomassie Brilliant Blue R250 (Bio-Rad) as described by Korn & Wright (1973).

Specimens

Three human lumbar spines from donors aged 61, 64 and 68 years, collected within 24h of death, were stored at −80°C tightly wrapped in layers of Saran wrap and aluminium foil until dissection, when the spines were thawed overnight at 4°C. The L1–L2 and L2–L3 discs were removed in the planes of the cartilaginous end-plates and freed of the surrounding perichondrium. The annulus fibrosus (tissue with fibrous rings through the full thickness of the disc) and the nucleus pulposus (centrally-located tissue free of such rings) were separated and the intervening transitional zone discarded. The annulus and nucleus of the two discs from each donor were pooled separately. Bovine nasal cartilage was obtained at the slaughterhouse within 1 h of death, kept on ice and processed without delay.

Preparation of fractions of proteoglycan aggregates

A mixture of aggregating and non-aggregating proteoglycans was prepared from each pool of tissue by mincing with a Latapie mill (A. H. Thomas, Philadelphia, PA, U.S.A.) and extracting for 24h at 4°C with 10ml (per g fresh wt.) of 4M-guanidinium chloride (grade 1, Sigma Chemical Co.) containing 0.05M-sodium acetate buffer, pH5.8, 10mM-sodium EDTA, 5mM-benzamidine hydrochloride, 0.1M-6-aminohexanoic acid (the latter two from Aldrich Chemical Co.) and 0.3mM-phenylmethane sulphonyl fluoride (Sigma), the latter four compounds called hereinafter 'proteinase inhibitors'. The extracts were dialysed for 24h at 4°C against two changes of 10vol. of 0.05M-sodium acetate buffer, pH5.8, containing proteinase inhibitors. CsCl (Kawecki Berylco Industries Inc., Revere, PA, U.S.A.) was added to the retentate to yield a density of 1.45g/ml and then centrifuged at 100000$g_{av.}$ using a type 60 Ti rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) for 48h at 20°C. The bottom one-quarter of the tube was collected by gravity (fraction A1; Heinegard, 1972), and dialysed against three changes of 10vol. of 0.5M-sodium acetate, pH5.8, containing 10mM-sodium EDTA, each for 24h at 4°C, and then stored at −20°C.

The residue remaining after extraction with 4M-guanidinium chloride was washed, digested with activated papain, then dialysed against water with a membrane expected to retain keratan sulphate (Spectra/Por 6, M_r cutoff 2000; Spectrum Medical Industries Inc., Los Angeles, CA, U.S.A.).

Hyaluronate (HA_{8000}) was added to the A1 fraction in amounts corresponding to 20μmol of hexuronate/mmol of A1 hexuronate and the solution was allowed to react overnight at 4°C. A 5ml portion, containing 15μmol of hexuronate, was applied at 4°C to a column (25mm × 750mm) of Sepharose CL-2B (Pharmacia) and eluted with the solvent at a rate of 18ml/h; 5ml fractions were collected and analysed for hexuronate. In later experiments, a 10ml sample was applied to a column (50mm × 750mm) of Sepharose CL-2B and 10ml fractions were collected. Excluded (A1-2B.V_0), included (A1-2B.V_i), and intermediate peaks were pooled separately and mixed with similar fractions of the same tissue sample from other runs. The pooled intermediate fractions from each donor were concentrated by ultrafiltration (type PM-10 membrane, Amicon Corp., Lexington, MA, U.S.A.), re-chromatographed, and the excluded and included fractions added to the corresponding pools; the intermediate fraction obtained upon re-chromatography was discarded. The pooled excluded and included fractions from each donor were concentrated by ultrafiltration to 1.5μmol of hexuronate/ml and stored at −20°C. The proteoglycan aggregate (A1-2B.V_0) was adjusted to 4M-guanidinium chloride and CsCl was added to a density of 1.45g/ml. After centrifugation at 100000g for 48h at 20°C, four equal fractions were collected from each tube by gravity, and are identified as A1-2B.V_0-D1 (bottom) to A1-2B.V_0-D4 (top).

Characterization of the A1-2B.V_0-D4 fractions

The A1-2B.V_0-D4 fraction was dialysed exhaustively against distilled water at 4°C and freeze-dried. A weighed sample was dissolved in 4M-

guanidinium chloride in 0.05M-sodium acetate buffer, pH5.8, and a portion containing about 40μg of protein was reduced and analysed by SDS/polyacrylamide-gel electrophoresis. A second portion of the solution was examined immunochemically in the laboratory of Dr. A. R. Poole, Shriner's Hospital for Crippled Children (Quebec), Montreal, PQ, Canada, as described by Roughley *et al.* (1982). Briefly, the samples were dialyzed against 0.125M-Tris/HCl buffer, pH6.8, containing 1g of SDS/l and applied without reduction to a 10% gel. After electrophoresis, the protein was transferred to nitrocellulose by using 200–300mA overnight and was reacted first with sheep antiserum to purified link protein from newborn human articular cartilage, then with peroxidase-labelled pig antibody to sheep immunoglobulin G. The peroxidase was detected by a colorimetric reaction. A third portion of the solution was added to 7ml of hyaluronate–Sepharose (Tengblad, 1979), dialysed against 10vol. of 0.5M-sodium acetate buffer, pH5.8, washed onto a column with the same buffer containing 1.0M-NaCl to remove contaminating protein, then eluted with 4M-guanidinium chloride in 0.05M-sodium acetate buffer, pH5.8, and stored at room temperature.

Preparation of ^{125}I-labelled 'link protein'

Proteoglycan aggregates (A1-2B.V_0) from donor I were dialysed exhaustively against distilled water and freeze-dried. A portion containing 6μmol of hexuronate was iodinated with 1mCi of Na^{125}I (1.4Ci/μmol; Amersham Corp., Oakville, ON, Canada) by using chloramine-T (Greenwood *et al.*, 1963). The product was fractionated by CsCl density-gradient centrifugation, as described above, and the A1-2B.V_0-D4 fraction was purified by binding to hyaluronate–Sepharose with subsequent elution, also as described. The specific activities were 2.57×10^7 and 2.62×10^7 c.p.m./mg of protein for annulus and nucleus, respectively. All radioactivity assays were done by using a Compugamma Universal Gamma Counter (LKB, Stockholm, Sweden) adjusted for optimum counting of ^{125}I.

Analytical agarose-gel chromatography

Samples of 1ml or less, containing at least 0.5μmol of hexuronate and/or 2×10^5 c.p.m. and equilibrated with 0.05M-Na_2SO_4 were applied to a column (6.5mm × 1600mm) of Sepharose CL-2B (Pharmacia) equilibrated with the same solvent, and pumped at 2.0–2.5ml/h; 1ml fractions were collected. In some experiments, the A_{206} of the eluate was recorded (Uvicord S, LKB Productor AB, Bromma, Sweden).

Viscometry

All measurements were made in a Manning semi-micro viscometer (size 100, model no. CMSMU, Cannon Instrument Co., State College, PA, U.S.A.) in a water bath maintained at 20.0±0.1°C. The solvent was 0.05M-sodium acetate buffer, pH5.8, containing 0.4M-guanidinium chloride if fraction A1-2B.V_0-D4 was present. The relative viscosity was calculated directly from outflow times, with the flow time for the solvent as denominator.

Treatment of aggregate and link with hyaluronidase

Hyaluronate was removed from proteoglycan aggregates (A1-2B.V_0) or from preparations of aggregating proteoglycan (A1-2B.V_0-D1) by treatment with a hyaluronidase prepared from *Streptomyces hyaluronolyticus* nov. sp. (specific activity 2000 turbidity-reducing units/mg; Seikagaku Kogyo Co., Tokyo, Japan). The enzyme (100 units) was dissolved in 1ml of 0.05M-sodium acetate buffer, pH5.8, and 10μl were added to 1ml of the proteoglycan solution containing 0.6μmol of hexuronate and incubated at 20°C for 4h.

Results

Preparation of proteoglycan aggregates

All discs were moderately degenerate (grade 2; Lewin, 1964), the usual finding for donors of this age (Pearce & Grimmer, 1983). The proteoglycans extracted from pools of annulus fibrosus with 4M-guanidinium chloride contained 76, 79 and 79% of the tissue hexuronate and from the nucleus pulposus 86, 92 and 86% for spines I, II, and III, respectively, confirming the greater ease of extraction of nucleus proteoglycan reported by Adams & Muir (1976). Associative CsCl density-gradient ultracentrifugation using a starting density of 1.45g/ml rather than 1.50g/ml (Emes & Pearce, 1975; Pearce & Grimmer, 1976; Stevens *et al.*, 1979) gave a higher yield of proteoglycan with little additional protein. The A1 fraction (Heinegard, 1972) contained 83±2 (6) % of the hexuronate of the extract (means±S.D., with the numbers of observations in parentheses, are given).

The aggregates and non-aggregable proteoglycans were separated by Sepharose CL-2B chromatography. The excluded fraction was 45.9±1.3 (7), 35.8±7.6 (5) and 44.0±1.5 (6) % of the proteoglycan from the annulus and 25.4±2.9 (7), 10.7±2.5 (4) and 30.0±2.6 (5) % of that from the nucleus for spines I, II and III, respectively. The higher content of aggregate in the annulus confirmed the results of Adams & Muir (1976), Stevens *et al.* (1979), and Lyons *et al.* (1981). Fractions judged to be mixtures of aggregating and

non-aggregating proteoglycan contained 16–30% of the hexuronate and were re-chromatographed. After addition of the appropriate fractions to the corresponding pools of the excluded and included fractions, the remaining overlap region represented less than 3% of the total hexuronate of each preparation.

Portions of the excluded (A1-2B.V_0) and included (A1-2B.V_i) fractions were applied separately to an analytical column of Sepharose CL-2B after the addition of hyaluronate sufficient to form aggregates (results not shown). All of the fraction A1-2B.V_0 appeared in the void volume; most of fraction A1-2B.V_i appeared in the included portion of the column. The small fraction that was excluded corresponded to the added hyaluronate. Similar results were obtained for both the annulus and nucleus of all spines. We concluded that fraction A1-2B.V_0 is proteoglycan aggregate free of non-aggregating proteoglycan and that fraction A1-2B.V_i is non-aggregating proteoglycan free of aggregate.

The A1 fraction contained enough hyaluronate to react with all the aggregable proteoglycans present, as reported by Hardingham & Adams (1976). After dissociative density gradient ultracentrifugation, fraction A1-2B.V_0-D4 contained an amount of protein approximately equal to A1-2B.V_0-D1, a much smaller proportion of carbohydrate and was enriched in hexose relative to hexuronate (results not shown).

Protein composition of 'link fraction'

The A1-2B.V_0-D4 fractions from nucleus pulposus and annulus fibrosus were compared with similar fractions from bovine nasal cartilage by using SDS/polyacrylamide-gel electrophoresis after reduction with mercaptoethanol (Fig. 1*a*). Two bands seen in all preparations were similar to those described by Baker & Caterson (1979) for link protein from bovine nasal cartilage. The annulus and nucleus each contained two additional more mobile bands similar to those reported by Mort *et al.* (1983) in preparations from mature articular cartilage. Several other proteins were present as well. The A1-2B.V_0-D4 fractions bound antibodies to the link protein of human newborn articular cartilage (Fig. 1*b*). Their mobilities on SDS/polyacrylamide-gel electrophoresis without reduction were those expected for proteins of M_r values 38000, 44200 and 50100 (results supplied by Dr. Poole). The hyaluronate-binding components of the mixture were purified by absorption to and elution from hyaluronate–Sepharose. After reduction, SDS/polyacrylamide-gel electrophoresis of the eluate showed two bands of M_r close to 43000 and two additional bands of M_r 20000 and 14000 (Fig. 1*c*).

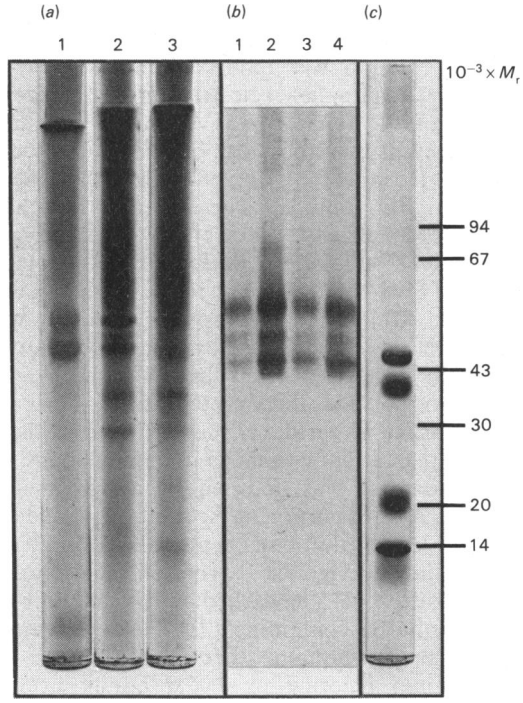

Fig. 1. *SDS/polyacrylamide-gel electrophoresis studies of A1-2B.V_0-D4 fractions*

(*a*) Reduced protein (40μg) from bovine nasal cartilage (1) and both annulus fibrosus (2) and nucleus pulposus (3) of spine I. (*b*) Preparations from nucleus pulposus (1) and annulus fibrosus (3) of spine II and from nucleus pulposus (4) and annulus fibrosus (2) of spine I were separated without reduction, transferred to nitrocellulose and stained after reaction with a sheep antibody to human link protein. (*c*) The preparation from nucleus pulposus of spine I was purified by binding and elution from hyaluronate–Sepharose; 40μg of protein was analysed after reduction. The M_r values of standards apply only to panel *c*.

Interaction of 'link fraction' with hyaluronate

The ability to bind hyaluronate is an essential characteristic of link proteins. The ^{125}I-A1-2B.V_0-D4 fraction had been prepared by absorption to and elution from hyaluronate–Sepharose. This procedure had selected from the aggregate preparation those components which bound to hyaluronate. Thus, the hyaluronate-binding components of A1-2B.V_0-D4 are demonstrated by the data of Fig. 1(*c*). Further experiments were undertaken to confirm this interaction. A mixture of both hyaluronate and the labelled A1-2B.V_0-D4 eluted as one peak in the void volume of a Sepharose CL-2B column, consistent with the formation of a complex (Fig. 2). However, since the free hyalur-

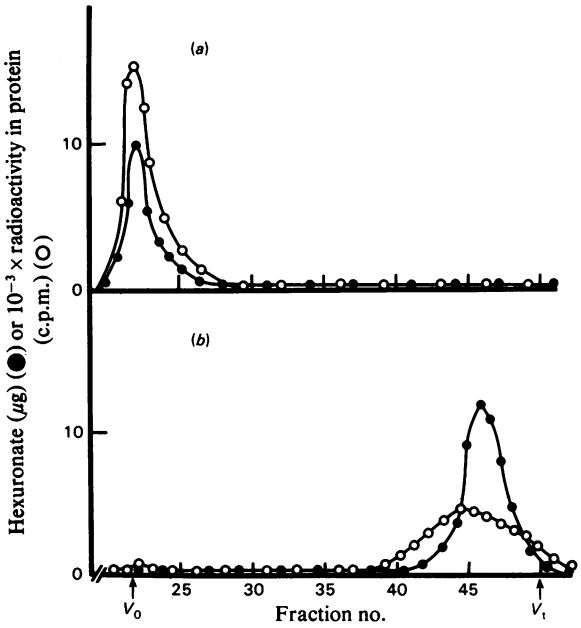

Fig. 2. *Interaction of 'link protein' with hyaluronate and a hyaluronate oligosaccharide*

^{125}I-A1-2B.V_0-D4 (1.4μg of protein containing 2.7×10^5 c.p.m.) and either (*a*) HA_{8000} (70nmol of hexuronate) or (*b*) HA_{14} (110nmol of hexuronate) in 4M-guanidinium chloride/0.5M-sodium acetate buffer, pH5.8, were dialysed overnight at room temperature against 8vol. of 0.05M-sodium acetate buffer, pH5.8, and applied to an analytical column of Sepharose CL-2B. Fractions were analysed for hexuronate (●) and assayed for radioactivity (○).

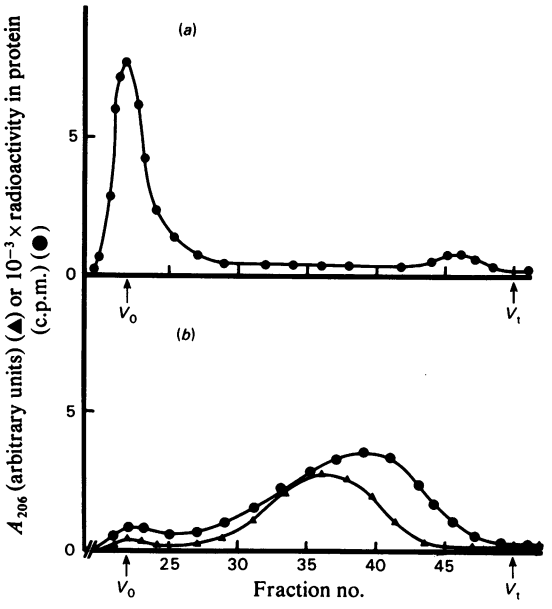

Fig. 3. *Interaction of 'link protein' with proteoglycan*

(*a*) ^{125}I-A1-2B.V_0-D4 (1.4μg of protein) and proteoglycan monomer (A1-2B.V_0-D1, 0.7μmol of hexuronate), both from the annulus fibrosus of donor I, were mixed and a portion was applied to an analytical column of Sepharose CL-2B. The fractions were analysed for radioactivity (●). (*b*) A second portion of the mixture was treated with *Streptomyces* hyaluronidase, dialysed against 4M-guanidinium chloride in 0.5M-sodium acetate buffer, pH5.8, then against the buffer alone and chromatographed similarly (●) The A_{206} was also measured (▲).

onate was sufficiently large to be excluded from the gel and the link protein may self-aggregate (Tang *et al.*, 1979), a hyaluronate oligosaccharide, HA_{14}, was substituted for the hyaluronate; both the hexuronate and the radioactivity appeared in the included volume of the column (Fig. 2). The hexuronate, which formed a much narrower peak than the radioactivity, is believed to represent a large excess of unbound oligosaccharide. Alternatively, the A1-2B.V_0-D4 may have bound preferentially to oligosaccharides larger than HA_{14} contained in the oligosaccharide preparation. Thus, the hyaluronate oligosaccharides, which by analogy with cartilage proteoglycan might be expected to form a 1:1 complex with the link protein, caused the protein to be eluted as a broad polydisperse peak. Since in the absence of oligosaccharides the protein is insoluble in the buffer, A1-2B.V_0-D4 could not be chromatographed separately for comparison. Thus, the A1-2B.V_0-D4 fraction formed a complex with hyaluronate of a size dependent upon the length of the hyaluronate molecule.

Interaction of 'link protein' with proteoglycan

A second essential characteristic of link protein is the ability to react with proteoglycan (Caterson & Baker, 1978). A mixture of aggregating proteoglycan monomer with ^{125}I-A1-2B.V_0-D4 was excluded from Sepharose CL-2B (Fig. 3), suggesting that enough hyaluronate was present to form aggregates. Accordingly, the mixture was treated with the highly specific *Streptomyces* hyaluronidase and dialysed under dissociative conditions to remove the hyaluronate oligosaccharides, then redialysed to associative conditions and chromatographed. The protein (radioactivity) and proteoglycan (A_{206}) were found in the included volume of the column; the latter eluted earlier, suggesting the presence of an excess of link protein. Treatment of the presumed aggregate with hyaluronidase had shifted the protein and the proteoglycan from the excluded to the included volume of the column, showing that the proteoglycan and link protein were associated.

Dissociation of hyaluronate–proteoglycan complex with hyaluronate oligosaccharides

Studies of cartilage have shown that a hyaluronate oligosaccharide containing ten or more monosaccharides can dissociate proteoglycan–hyaluronate aggregates (Hardingham, 1979). Hyaluronate added to aggregating proteoglycan from disc produced a marked increase in relative viscosity (Fig. 4). Only enough hyaluronate was added to assure complete formation of aggregates. A slight excess of various oligosaccharides was added to similar mixtures. HA_{10} reduced the specific viscosity rapidly during the initial 2h, falling in 20h to 30–40% of the difference between monomer and aggregate, but with HA_8 in higher concentrations the values remained at 80–90% of the initial values (Fig. 4). HA_6 and HA_4 behaved similarly to HA_8 (results not shown). The A1-2B.V_0-D1 fractions prepared from annulus fibrosus and nucleus pulposus of all the spines behaved similarly. Thus, a hyaluronate oligosaccharide of five or more disaccharide units was capable of dissociating the hyaluronate–proteoglycan from disc aggregate.

Effect of 'link protein' on the stability of the aggregate

Link protein inhibited the dissociation of the aggregate of hyaline cartilage proteoglycan by hyaluronate oligosaccharides (Hardingham, 1979). After adjusting the concentrations of mixtures of aggregating and non-aggregating proteoglycans from annulus, nucleus and cartilage to give comparable viscosities under associative conditions, HA_{10} caused the viscosity to fall rapidly for 2h, then more slowly over a total of 20h (Fig. 5). The viscosity of aggregates treated with *Streptomyces* hyaluronidase was used to assess the effect of complete dissociation (cf. Fig. 4). The results were expressed as a percentage of the difference between the viscosities of aggregate (100%) and those of the hyaluronidase-treated aggregate (0%). After the addition of HA_{14} to the fractions from disc, the viscosity increment due to aggregate fell to 30–40% of its initial value in 20h, whereas for the fractions from cartilage the increment fell only to 80% (Fig. 5). In other experiments, the effect of HA_{10} on the viscosities of a mixture of A1-2B.V_0-D4, A1-2B.V_0-D1 and HA_{8000} was compared with its effect on a mixture of A1-2B.V_0-D1 and HA_{8000}; no differences were noted (results not given). Thus, the link protein did not appear to stabilize aggregates from disc, in contrast to those from cartilage.

Discussion

The proteins associated with the aggregate of disc proteoglycans resemble in many respects the link proteins from hyaline cartilage. They bind to hyaluronate, a penta-disaccharide being required to displace macromolecular hyaluronate (Hardingham, 1979; Tengblad, 1981). They also bind to

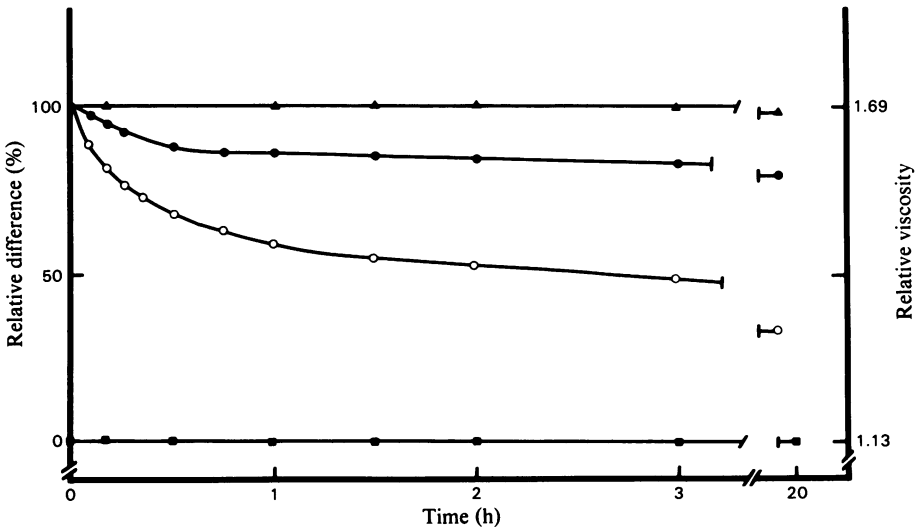

Fig. 4. *Effect of hyaluronate oligosaccharides on the interaction between hyaluronate and aggregating proteoglycans*
Fraction A1-2B.V_0-D1 (0.6μmol of hexuronate) from the annulus fibrosus of donor I was incubated for 20h at 4°C in 0.05M-sodium acetate buffer, pH5.8, with HA_{8000} (36nmol of hexuronate). HA_8 (60nmol of hexuronate, ●), HA_{10} (42nmol of hexuronate, ○), or an equivalent volume of buffer (▲) were added and the relative viscosities were measured at intervals. A control treated with added hyaluronidase (■) was also assayed.

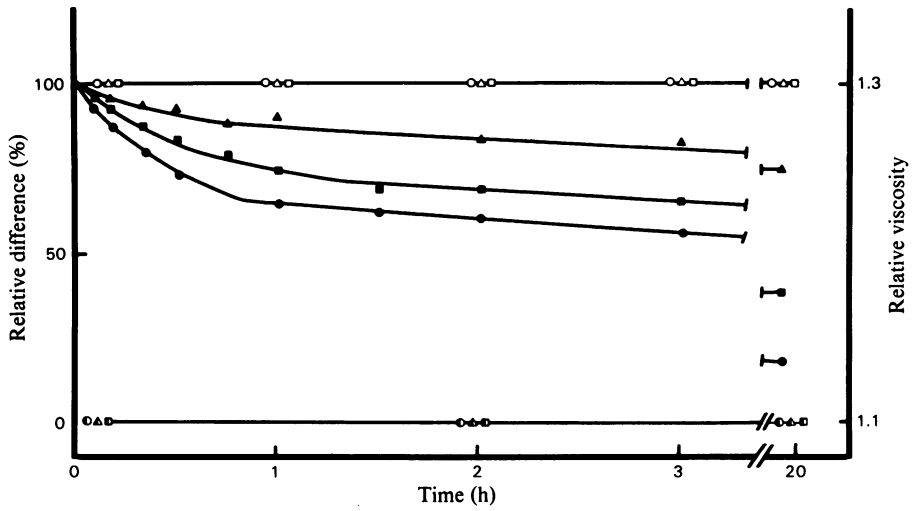

Fig. 5. *Effect of hyaluronate oligosaccharides on the viscosities of aggregates from annulus fibrosus, nucleus pulposus and bovine nasal cartilage*

A1 fractions prepared from annulus fibrosus (0.6μmol of hexuronate/ml; ●, ○), nucleus pulposus (0.6μmol of hexuronate/ml; ■, □), both from donor I, or from nasal septa (1.1μmol of hexuronate/ml; ▲, △) in 0.5M-sodium acetate buffer, pH5.8, were mixed with either HA_{10} (70nmol of hexuronate/μmol of A1 hexuronate; solid symbols) or an equivalent volume of buffer (open symbols). Other portions of the A1 fractions were treated with *Streptomyces* hyaluronidase (◐, ◧, ◭ for annulus, nucleus and nasal septum, respectively).

the proteoglycan (Caterson & Baker, 1978; Tengblad, 1981). The M_r values resemble those reported for cartilage link (Hascall & Heinegard, 1974*b*; Bonnet *et al.*, 1978; Baker & Caterson, 1977; Tengblad, 1979, 1981) and they react with antibodies to link protein prepared from human articular cartilage (Roughley *et al.*, 1982). In each of these properties the aggregate-associated protein of the intervertebral disc resembles the link protein of hyaline cartilage.

Surprisingly, the ability of the disc protein to stabilize the aggregate was much less than that of the cartilage link protein. The present data do not provide a clear explanation of this observation: the quantity of protein added may have been insufficient to stabilize all the aggregate, an excess of hyaluronate may have inhibited co-operative binding (Tengblad, 1981), the protein may have been degraded *in situ* or during its isolation, or the dissociation constants for the protein–hyaluronate and protein–proteoglycan interactions may be higher for disc proteoglycans than those of cartilage. The presence of materials of low M_r after reduction with mercaptoethanol corresponded strikingly with the similar findings of Mort *et al.* (1983) for bovine articular cartilage. If the fragments of low M_r represent degradation products of the link protein, they may be less effective in stabilizing the aggregate than their precursor. Resolution of these problems would require a larger supply of highly purified reagents than was available for the present work.

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