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Short Communications

Multiple Aggregation Factors in Cartilage Proteoglycan

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Proteoglycan as isolated from bovine nasal septa is a partially aggregated complex containing linking factors. Two essential factors can be separated from proteoglycan subunits and resolved in a CsCl density gradient. Chondroitinase digestion of the purified proteoglycan subunits does not interfere with their aggregation when the linking factors are added.

Proteoglycans of hyaline cartilage are composed of chondroitin sulphate and keratan sulphate covalently linked to protein. In bovine nasal septum the molecules have an average molecular weight of 2.5×10^6 (Hascall & Sajdera, 1970) and contain about 7% of protein, to which the many polysaccharide chains are attached in a brush-like structure. The chemical and physical properties of this material have received much attention (Dunstone & Franek, 1969; Hascall & Sajdera, 1969, 1970; Rosenberg *et al.*, 1970; Hascall & Riolo, 1972; Tsiganos *et al.*, 1972; Gregory & Hascall, 1972; Keiser *et al.*, 1972).

In the tissue, proteoglycans exist to a large extent as aggregate complexes of much higher molecular weight (Dunstone & Franek, 1969) that can be disaggregated and extracted from the tissue in high yields by dissociative solvents such as 4M-guanidinium chloride. When the ionic strength of the extract is lowered to associative conditions, about 60% of the proteoglycans reaggregate (Sajdera & Hascall, 1969). This product, monomers and complexes together, can then be purified by equilibrium centrifugation in a CsCl density gradient to yield a preparation called 'proteoglycan complex'. When this is recentrifuged in a CsCl density gradient in a dissociative solvent, 4M-guanidinium chloride, monomeric proteoglycan, called 'proteoglycan subunit', is found at the bottom of the gradient. A fraction, referred to as 'glycoprotein link', which represents about 3% by weight of the complex, is recovered at the top of the gradient. Subunit prepared in this manner can no longer aggregate in associative solvents unless the link factors are again added (Hascall & Sajdera, 1969). The phenomenon has been observed by viscometry and analytical ultracentrifugation (Hascall & Sajdera, 1969) and by chromatography (Tsiganos *et al.*, 1971).

The present report deals with more detailed observations on the link fraction and on the reaggregation of proteoglycan subunit. The initial density for a dissociative CsCl density gradient has usually been 1.50g/ml. With a lower initial density and a more careful sectioning of the resulting gradient, however, the link fraction can be resolved into two functional classes of molecules with different buoyant densities. Both of them must be added to subunit to form aggregate complexes that can be seen in the ultracentrifuge.

Materials and methods

Proteoglycan was extracted from bovine nasal septa with 4M-guanidinium chloride buffered at pH 5.8 with 0.05M-2-(*N*-morpholino)ethanesulphonic acid. Unless otherwise stated all subsequent experiments contained this buffer. The complex was purified by sedimentation in a CsCl density gradient after dialysis to associative conditions (0.4M-guanidinium chloride). Proteoglycan subunit and link factors were made from the complex by sedimentation in a CsCl density gradient in the presence of 4M-guanidinium chloride. Details of the procedures have been described by Sajdera & Hascall (1969) and Hascall & Sajdera (1969). Equilibrium sedimentation was done for 65h at 22°C in a Beckman–Spinco type 50 rotor at $86000g_{av.}$ or in a type SW50.1 rotor at 131000g. Densities were measured in a calibrated 1ml constriction pipette at 23°C. Guanidinium chloride was Ultrapure grade from Schwarz/Mann (Orangeburg, N.Y., U.S.A.), and CsCl was Radio Tracer grade from Harshaw Chemical Co. (Cleveland, Ohio, U.S.A.). Human umbilical-cord hyaluronic acid was a standardized preparation provided by Dr. J. A. Cifonelli.

Reconstitution experiments were done by mixing equal volumes of fractions obtained from the CsCl density gradients, diluting the proteoglycan content tenfold with 4M-guanidinium chloride and dialysing overnight to achieve 0.4M-guanidinium chloride. The proteoglycan concentrations were then adjusted on the basis of the uronic acid content (Hascall & Sajdera, 1970). Analytical ultracentrifugation was done at room temperature at 40000rev./min in a

Beckman–Spinco model E ultracentrifuge with a double-sector 30mm cell. Photographs were made with schlieren optics and a bar angle of 60°. Timing was begun on reaching two-thirds of full speed.

Digestion of proteoglycan complex or subunit with chondroitinase ABC (Miles Laboratories, Kankakee, Ill., U.S.A.) was carried out and assayed as described by Hascall *et al.* (1972). After digestion, preparations were dialysed against 0.4M-guanidinium chloride and buffer as with all other samples. The actual concentration was not measured, but appropriate dilution for analysis in the ultracentrifuge was calculated on the basis of the original proteoglycan content and the fact that about 80% of the weight is lost on digestion.

Results and discussion

Fig. 1(*a*) shows the sedimentation pattern of proteoglycan complex, and Fig. 1(*b*) shows that of proteoglycan subunit. The dissociative gradient used to separate subunit from the link components is diagrammed in Fig. 2(*a*). The fractions used in earlier work (Hascall & Sajdera, 1969) were taken from the portions of the gradient that had densities greater than 1.57g/ml and less than 1.45g/ml, or approximately the bottom two fractions for subunit and the upper two for link. The recombination experiments illustrated in Fig. 1, however, were made with fraction 6, which contains 85–90% of the subunit molecules, and fractions 1 and 2. Addition of fraction 1 or of fraction 2 to the subunit did not yield aggregate complexes (Fig. 1*c*), even if large amounts of link were used. When both were added, aggregation occurred (Fig. 1*d*). In the examples shown, the fractions were mixed in dissociative conditions and then dialysed. Fig. 1(*e*) indicates that the fractions can be dialysed against an associative solvent, 0.4M-guanidinium chloride, before mixing and still retain their ability to form aggregates.

Proteoglycan complex or subunit can be digested with bacterial chondroitinases to remove the chondroitin sulphate, but not keratan sulphate, from the macromolecules. Analyses in the ultracentrifuge show that chondroitinase-treated complex still contains aggregate (Fig. 1*f*). The aggregate component in chondroitinase-treated complex dissociates in 4M-guanidinium chloride and reassociates when the

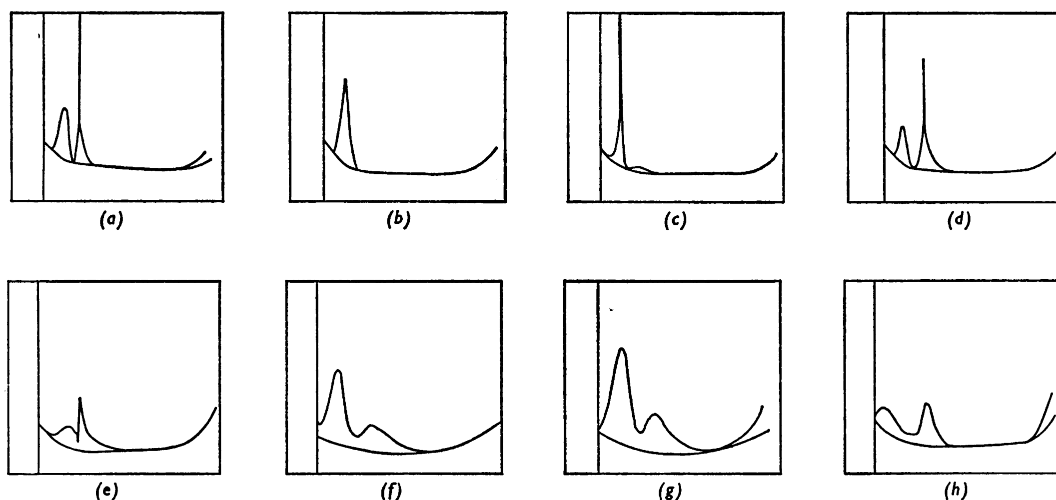

Fig. 1. *Schlieren patterns of proteoglycan preparations*

All samples are under associative conditions in order to show any aggregation. The meniscus is at the left, the monomer peak next and the aggregate at the right. (*a*) Proteoglycan complex after purification in a CsCl density gradient, concn. 1.1mg/ml, time 12min. (*b*) Proteoglycan subunit showing monomer peak only, concn. 1.1mg/ml, time 12min. (*c*) Subunit combined with either link 1 or link 2; concn. 1.25mg/ml; time 12min. (*d*) Subunit combined with both link 1 and link 2; concn. 1.25mg/ml; time 12min. (*e*) Subunit and links dialysed against 0.4M-guanidinium chloride and then mixed; concn. 1.1mg/ml; time 12min. (*f*) Complex digested with chondroitinase ABC, concn. equivalent to 16.5mg of proteoglycan/ml; time 36min. (*g*) Same as (*f*) after two cyles of dissociation and association. (*h*) Chondroitinase-digested subunit combined with both link 1 and link 2; concn. equivalent to 9.6mg of proteoglycan/ml; time 18min.

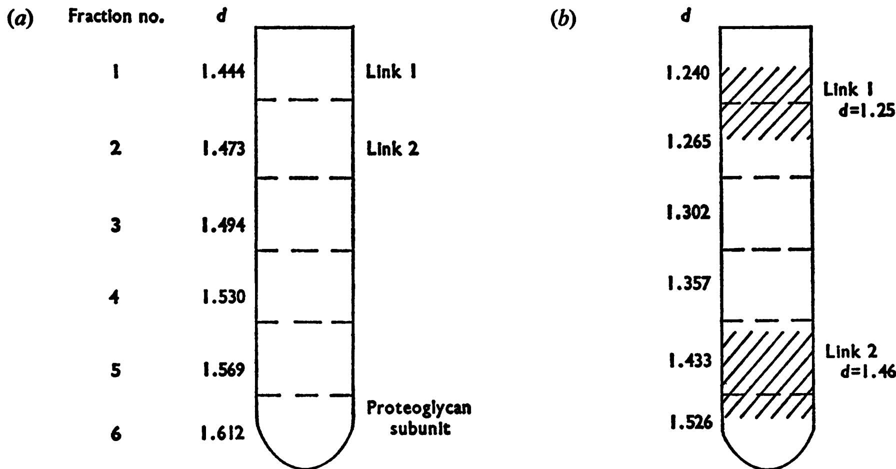

Fig. 2. *Equilibrium sedimentation in CsCl density gradients*

(*a*) Sedimentation of proteoglycan complex in 4M-guanidinium chloride for the separation of link fractions and subunit; initial density 1.52g/ml; angle rotor type 50. (*b*) Second sedimentation of link factors in the same solvent; initial density 1.353g/ml; swinging-bucket rotor type SW 50.1. Link fractions were located by their activity in promoting aggregation of subunit as observed in the analytical ultracentrifuge.

ionic strength is lowered again to 0.4M-guanidinium chloride (Fig. 1*g*) (Gregory & Hascall, 1972). Chondroitinase-treated subunit contains only the slower-sedimenting peak (Hascall *et al.*, 1972), and this forms aggregates when mixed with the link fractions (Fig. 1*h*). It is thus apparent that the chondroitin sulphate portion of the proteoglycans does not participate directly in aggregate formation.

The link factors were recentrifuged in a dissociative gradient with a lower initial density (Fig. 2*b*). Recombination experiments with these fractions showed that maximum reaggregation occurred when subunit was mixed with material from two zones, with densities about 1.25g/ml and 1.46g/ml. It may be therefore that the link 1 component is a protein or glycoprotein, but link 2, because of its density, is more likely to be proteoglycan or polysaccharide.

While this work was in progress, Hardingham & Muir (1972) described the interaction of proteoglycan subunit from pig larynx with hyaluronic acid isolated from various sources. The stoicheiometry of the interaction suggested that 10–30 proteoglycan molecules could bind to a single molecule of hyaluronate. This interaction showed the same instability to high guanidinium chloride concentrations and low pH that bovine nasal complex exhibits (Hascall & Sajdera, 1969). It was therefore important to determine the buoyant density of hyaluronic acid in a CsCl density gradient containing 4M-guanidinium chloride like that used for characterizing the link fractions. Human umbilical-cord hyaluronic acid was found to have a density of 1.46g/ml, exactly like link 2. It is thus possible that link 2 is hyaluronic acid, although much more direct evidence is required.

It is noteworthy that the buoyant density of hyaluronic acid in CsCl depends on the nature of the cations present. The value of 1.46g/ml in 4M-guanidinium chloride may be compared with the reported value of 1.66g/ml in its absence (Silpananta *et al.*, 1967).

The link factors described here have not yet been prepared in sufficient quantity or purity to permit chemical characterization. At present the linking activity cannot be attributed to any specific components of the fractions. The density gradients separate the active species well, but there is evidently a continuum of proteoglycan-like material throughout the gradient that obscures the chemical identity of the link factors.

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