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# Assembly of proteoglycan aggregates in cultures of chondrocytes from bovine tracheal cartilage

Sven BJÖRNSSON and Dick HEINEGÅRD
*Department of Physiological Chemistry, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden*

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The assembly of proteoglycan aggregates in chondrocyte cell cultures was examined in pulse–chase experiments with the use of [$^{35}$S]sulphate for labelling. Rate-zonal centrifugation in linear sucrose density gradients (10–50%, w/v) was used to separate the aggregated proteoglycans from monomers and to assess the size of the newly formed aggregates. The proportion of aggregates stabilized by link protein was assessed by competition with added exogenous aggregate components. The capacity of the proteoglycans synthesized in culture to compete with exogenous nasal-cartilage proteoglycans for binding was studied in dissociation–reassociation experiments. The results were as follows. (*a*) The proteoglycan monomers and the hyaluronic acid are exported separately and combined extracellularly. (*b*) The size of the aggregates increases gradually with time as the proportion of monomers bound to hyaluronic acid increases. (*c*) All of the aggregates present at a particular time appear to be link-stabilized and therefore not dissociated by added excess of nasal-cartilage proteoglycan monomer or hyaluronic acid oligomers. (*d*) The free monomer is apparently present as a complex with link protein. The monomer–link complexes are then aggregated to the hyaluronic acid. (*e*) The aggregates synthesized *in vitro* and the nasal-cartilage aggregates differ when tested for link-stabilization by incubation at low pH. The aggregates synthesized *in vitro* were completely dissociated whereas the cartilage proteoglycans remained aggregated. The results obtained from dissociation–reassociation experiments performed at low pH indicate that the proteoglycan monomer synthesized *in vitro* does not bind the hyaluronic acid or the link protein as strongly as does the nasal-cartilage monomer.

The biological function of cartilage as a shock-absorber is dependent on the molecular organization of the major components of the tissue, proteoglycans and collagen. The proteoglycan aggregates occupy large domains inside the collagen network and immobilize the water in the tissue. Proteoglycan aggregates may contain 50–100 proteoglycan monomers non-covalently bound to hyaluronic acid (for references see Hascall & Heinegård, 1979). Each aggregate, when extended in solution, has a hydrodynamic volume approaching the volume of the chondrocyte. Two central problems pertaining to the biology of cartilage are therefore how the chondrocyte handles these large molecules and how proteoglycans move through the matrix to be deposited at a required site. A primary question is whether the aggregates are assembled in the cell or in the matrix.

In the aggregate the proteoglycan monomers bind to hyaluronic acid by the hyaluronic acid-binding region of the protein core of the monomer. The interaction is stabilized by link protein. The aggregates may thus be link-stable or link-free. The proteoglycan monomer in the link-free aggregate is open to exchange with free monomer or hyaluronic acid, whereas the monomers in link-stable aggregates appear irreversibly bound to the hyaluronic acid. Thus, when either component of the aggregate is added in large excess, the monomers in link-free aggregates are displaced when the new equilibrium takes place (Kimura *et al.*, 1979). Kimura *et al.* (1979), using rat chondrosarcoma cells in monolayer cultures, have shown that link-stable aggregates are formed extracellularly, probably via a monomer link-protein precursor (Kimura *et al.*, 1980).

In a previous paper (Björnsson & Heinegård, 1981*a*) we described the procedure used to isolate and culture foetal bovine chondrocytes. The cells, even in the absence of serum, synthesized a cartilage



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proteoglycan with all typical features (Björnsson & Heinegård, 1981*b*). The proteoglycans were present in the medium as aggregates of similar size to those from the tracheal tissue. Both link proteins present in aggregates from the tissue were identified in the aggregates from the cell cultures.

The aim of the present investigation was to monitor the assembly of such proteoglycan aggregates in cultures of foetal bovine chondrocytes in the absence of exogenous proteins, which might affect the aggregation. Rate-zonal centrifugation in sucrose density gradients was used to separate aggregates and monomers and to determine the size of the aggregates.

## Experimental

### *Materials*

Collagenase CLS II was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Ham's F12 medium was from GIBCO (Grand Island, NY, U.S.A.). Benzoylpenicillin was from Astra (Stockholm, Sweden). Streptomycin sulphate was from Merck (Darmstadt, Germany). Polypropylene vials (1.5ml, no. 3810, and 2.2ml, no. 3812) were obtained from Eppendorf (Hamburg, Germany). Carrier-free [$^{35}$S]sulphate was from The Radiochemical Centre (Amersham, Bucks., U.K.). Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hyaluronic acid (Healon) was a gift from Pharmacia Fine Chemicals (Uppsala, Sweden). Dextran sulphate and Sepharose 2B were purchased from Pharmacia. Instagel was from Packard Instrument Co. (La Grange, IL, U.S.A.).

Proteoglycan aggregates (NC-A1 fraction) and monomers (NC-A1-D1 fraction) were prepared from bovine nasal cartilage as described previously (Heinegård, 1972; Heinegård & Hascall, 1979). Hyaluronic acid oligomers were isolated from partial digests of hyaluronic acid with testicular hyaluronidase by gel chromatography on Sepharose G-50 as described previously (Hascall & Heinegård, 1974). $HA_{14}$ fraction denotes an oligomer of hyaluronic acid containing 14 monosaccharides.

### *Preparation of cell cultures*

Chondrocytes were isolated from foetal bovine tracheas as described previously (Björnsson & Heinegård, 1981*a*). After incubation for 1–2 days at 37°C, the cells were collected by centrifugation and suspended in Ham's F12 medium (without streptomycin sulphate). The cell suspension was pipetted into Eppendorf vials and incubated at $10 \times 10^6$ cells/ml on an Adams nutator with orbital motion for 1h before being labelled with [$^{35}$S]sulphate.

### *Pulse experimental protocol*

The cells ($20 \times 10^6$ in 2ml) were pulsed with [$^{35}$S]sulphate (5$\mu$Ci in 50$\mu$l) for times between 2.5 and 60min. The pulse was terminated by pelleting the cells for 30s in an Eppendorf centrifuge (model 5412) operated with low voltage (90V).

The medium was rapidly withdrawn with a syringe, and the cell pellet was resuspended in 1ml of fresh non-radioactive medium and again pelleted as above. All the cell pellets were frozen in liquid $N_2$ and kept at −60°C until extracted.

The cells were extracted with 1ml of 4M-guanidinium chloride / 50mM-sodium acetate buffer, pH5.8, containing 10mg of NC-A1 fraction/ml for 14h at 4°C. After addition of 5ml of the same solution to the extract, any particulate material was pelleted by centrifugation at $100000 g_{av.}$ for 1h. The supernatants were dialysed against 10vol. of 50mM-sodium acetate buffer, pH5.8, containing proteinase inhibitors (0.1M-6-aminohexanoic acid, 10mM-$Na_2EDTA$ and 5mM-benzamidine hydrochloride). Associative density-gradient centrifugation performed as described previously (Heinegård, 1972; Heinegård & Hascall, 1979) was used to isolate the proteoglycans from the reassociated cell extract. The A1 fractions were chromatographed on a Sepharose 2B column (150cm × 0.9cm) eluted with 0.5M-sodium acetate buffer, pH7.0. Fractions (1.50ml) collected were analysed for radioactivity and uronic acid contents (Heinegård, 1973).

### *Pulse–chase protocol*

The cells ($6 \times 10^6$ in 0.6ml) were pulsed with [$^{35}$S]sulphate (12$\mu$Ci in 25$\mu$l) for 15min. The pulse was terminated by the addition of 600$\mu$l of fresh unlabelled medium containing 20mM-$Na_2SO_4$. The cells were pelleted as described above and the medium was discarded. The pellets were suspended in 1200$\mu$l of fresh medium containing 10mM-$Na_2SO_4$ and again pelleted. Finally, the cells were resuspended in 600$\mu$l of the same medium (Scheme 1). The time between the end of the pulse and the start of the chase was approx. 1.5min. By this procedure the amount of unincorporated [$^{35}$S]-sulphate remaining in the medium was decreased to below 0.02% of the concentration during the pulse. The cells were incubated as above, and the chase was continued for the times stated in the particular experiment. At the end of the chase the cells were pelleted and the medium was withdrawn. The distribution of [$^{35}$S]sulphate was studied in sucrose gradients, either immediately at the end of the chase or after incubation with added exogenous monomer or hyaluronic acid or hyaluronic acid oligosaccharide. In some experiments dissociation–reassociation of the proteoglycan aggregates was performed as described below.

$6 \times 10^6$ cells in 600 $\mu$l/Eppendorf vial

↓ Pulse: 15 min, 20 $\mu$Ci of [$^{35}$S]sulphate/ml

End of pulse: 600 $\mu$l of medium containing 20 mM-$Na_2SO_4$ is added. The mixture is centrifuged for 30 s. The medium is removed and the cells are suspended in 1200 $\mu$l of fresh medium. This procedure is repeated once.

↓ Chase: 15 min to 6 h

The mixture is centrifuged for 30 s, and 50 $\mu$l of the medium is layered on top of a 10–50% sucrose density gradient. This is centrifuged at 50000 rev./min for 36 min. The tube is frozen and sliced into 30 fractions.

Scheme 1. *Protocol used for pulse–chase experiments*
See the text for details.

Two pulse–chase experiments are discussed in the text. The first experiment was performed with either 15 min or 24 h of chase after the 15 min pulse. At the end of the 15 min chase, one sample (200 $\mu$l) was incubated at 37°C for 24 h in the absence of cells. Another sample (200 $\mu$l) was immediately layered on top of a sucrose gradient prepared in advance and centrifuged at 240000 $g_{av.}$ for 3 h. The two samples incubated with and without cells for 24 h were centrifuged as above.

The second experiment was performed with 15, 30, 45, 90, 180 or 360 min of chase after the 15 min pulse, and analysed as described below.

*Size of aggregates.* Portions (50 $\mu$l) of the samples were layered on sucrose density gradients and centrifuged at 240000 $g_{av.}$ for 36 min immediately after the chase. After sedimentation for only 36 min, the proteoglycan monomer is only partly resolved from the unincorporated label at the top of the gradient. Therefore the amount of radioactivity incorporated into proteoglycans was obtained from an experiment where samples of the same medium were dialysed so that the unincorporated label was removed before centrifugation in the sucrose density gradient. The proportion of monomers that had aggregated was determined as the radioactivity sedimenting as aggregates (fractions 1–23 in Fig. 3) divided by the total proteoglycan radioactivity, determined as the radioactivity sedimenting as monomers (fractions 15–25 in Fig. 5*e*) after dissociation–reassociation of the sample with excess of nasal-cartilage proteoglycan monomer, performed as described below.

*Stability of the aggregates.* Portions of the same samples were incubated for 24 h at 4°C as follows: (1) with added NC-A1-D1 fraction at 3 mg/ml, (2) with $HA_{14}$ fraction at 0.2 mg/ml and (3) with hyaluronic acid at 0.2 mg/ml; they were then centrifuged at 240000 $g_{av.}$ for 36 min. The proportion of aggregated proteoglycans was calculated as described above.

*Dissociation–reassociation.* To other portions of the same samples was added an equal volume of 8 M-guanidinium chloride to dissociate the proteoglycan aggregates. Subsequent dialysis to associative conditions was performed in a constantly moving micro dialysis chamber (Franzén *et al.*, 1981), which allows dialysis of small volumes without changes of volume. Reassociation was performed as follows: (1) without added exogenous proteoglycans, (2) with added NC-A1 fraction at 3 mg/ml and (3) with added NC-A1-D1 fraction at 3 mg/ml. Dialysis was performed overnight against a solution containing the proteinase inhibitors discussed above.

*pH-stability of the aggregates.* Samples were adjusted to pH 4.0 by incubating the medium with 1 vol. of 0.1 M-citric acid, pH 2.5, for 24 h before sucrose-density-gradient centrifugation at pH 4.0.

### *Analytical methods*

Rate-zonal centrifugations of proteoglycans were done in sucrose density gradients. Linear sucrose density gradients (3.6 ml) with 10–50% (w/v) sucrose in 0.5 M-NaCl / 5 mM-sodium phosphate buffer, pH 7.0, (or 0.5 M-NaCl / 1 mM-citric acid buffer, pH 4.0) were prepared in 4.2 ml MSE polycarbonate tubes. The samples (50–200 $\mu$l) were carefully layered on top of the gradient. The gradients were centrifuged in a swing-out MSE $6 \times 4.2$ ml rotor at 240000 $g_{av.}$. By 36 min of centrifugation, the aggregates sedimented approximately halfway down the gradient, whereas the monomers essentially remained at the top of the gradient. Alternatively, the gradients were centrifuged for 3 h, in which case the monomers were recovered at a position halfway down the gradient, whereas aggregates had sedimented to the bottom.

The gradients were frozen and sliced in order to achieve a good recovery of the material sedimenting to the bottom of the tube. The gradients were frozen in an aluminium block, and 200 $\mu$l of 10% (w/v) sucrose containing 0.01% Bromophenol Blue was frozen on top of the frozen gradients. They were mounted in a vice and chilled with solid $CO_2$, and the bottom 2 mm of the tube was cut off with a saw. A Repette (Jencon) stepwise-dispensing syringe was fixed (with the barrel removed) to the vice with the Teflon piston inserted into the centrifuge tube, facing the frozen Bromophenol Blue solution. The frozen gradient was then stepwise expelled out of the tube, and 2 mm slices were cut with a scalpel. The top layer of 0.01% Bromophenol Blue in 10% sucrose was used to separate the top of the gradient from the

piston to assure reproducible slicing of the top. The Bromophenol Blue colour was included to detect any thawing of the gradient during slicing. The slices were analysed for radioactivity. In some experiments the gradients were emptied from the bottom by a glass capillary connected to a peristaltic pump via an LKB Uvicord spectrophotometer to measure the absorbance at 206nm, i.e. the proteoglycan carrier. When required, 0.12ml fractions were collected and analysed for radioactivity. Radioactivity was measured in a Packard 2650 scintillation counter, with Instagel as the scintillation medium. The sample volume was increased to 2ml by addition of water to prevent precipitation of the sucrose on addition of 3ml of Instagel.

## Results and discussion

### *Incorporation of [$^{35}$S]sulphate*

In a previous study, serum-free cultures of foetal bovine chondrocytes were used to monitor the incorporation of [$^{35}$S]sulphate into glycosaminoglycans over 6 days (Björnsson & Heinegård, 1981*a*). Most of the $^{35}$S-labelled glycosaminoglycans were recovered soluble in the medium, and only a minor portion (5% of total) was associated with the cells. The times selected were too sparse to allow any conclusions with regard to the accumulation of the cellular pool. In order to study whether one population with time transforms into another, pulse–chase experiments are usually applied. An experiment with continuous pulse was performed to determine the minimal length of the pulse needed to equilibrate the cells with [$^{35}$S]sulphate and to obtain a constant rate of export of the proteoglycans. The cells were separated from the medium at the times indicated in Fig. 1 and extracted with 4M-guanidinium chloride. Proteoglycans were then prepared from the medium and the extract by associative gradient centrifugation as described in the Experimental section. The cellular pool was equilibrated with [$^{35}$S]sulphate within 10min of the pulse, though it again increased slowly at later times (Fig. 1). This second phase probably reflects the accumulation of the pericellular pool at the surface of the cells. The medium pool increased linearly from 10min, coinciding with the equilibration of the cellular pool. Pulse–chase experiments were therefore performed with a pulse of 15min in order to saturate the cellular pool with [$^{35}$S]sulphate.

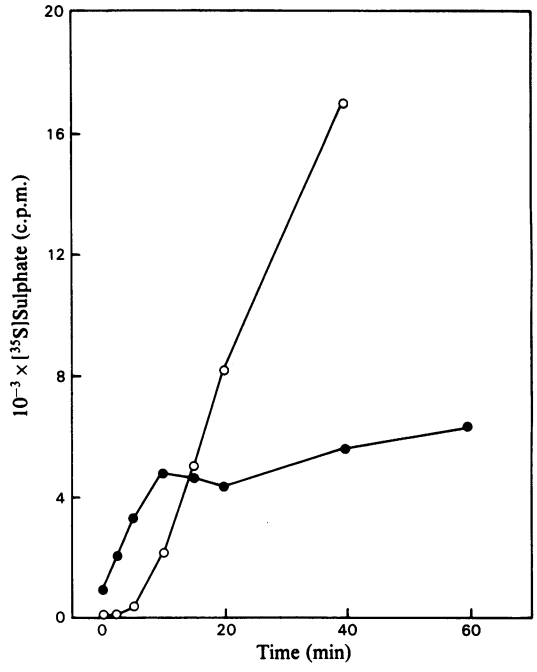


Fig. 1. *Incorporation of [$^{35}$S]sulphate into proteoglycans isolated by CsCl-density-gradient centrifugation*
Experimental details are given in the text. ○, A1 fractions prepared from the culture medium; ●, A1 fractions prepared from the 4M-guanidinium chloride extract of the cells.

### *Aggregatability of monomers*

The 4M-guanidinium chloride extract of the cells from the continuous-pulse experiment discussed above was reassociated in the presence of nasal cartilage proteoglycan aggregates as described in the Experimental section. The bottom fractions from the associative CsCl density gradients were chromatographed on Sepharose 2B (results not shown). Already at 15min of a [$^{35}$S]sulphate pulse approx. 75% of the radioactive proteoglycan monomers were eluted in the void volume. The 4M-guanidinium chloride extract of the cells, which constitutes 95% of the cellular [$^{35}$S]sulphate-labelled material, contains proteoglycan monomers derived from the intracellular pool. On reassociation in the presence of nasal cartilage proteoglycan aggregates the radioactive cell proteoglycan monomers and the non-radioactive nasal cartilage proteoglycan monomers will form mixed aggregates. Thus the intracellular pool consists of molecules that already have the capability of interacting with hyaluronic acid, even though binding might not occur until a later stage.

### *Localization of the site of aggregation*

In order to determine the site of aggregation, whether extra- or intra-cellular, a pulse–chase experiment with [$^{35}$S]sulphate was performed with either 15min or 24h of chase. At the end of the chase the proportion of aggregates and monomers was determined by rate-zonal centrifugation in

sucrose density gradients as described above. The gradients were centrifuged at 240000$g_{av.}$ for 3h. After 15min of chase (Fig. 2), only 2.6% of the incorporated radioactivity was recovered at the bottom of the tube sedimenting as aggregates, and the rest was in the middle of the tube at the position of monomers. The unincorporated label remained at the top of the gradient and is not included in the calculations. After 24h of chase the proportion of [$^{35}$S]sulphate in aggregates was 95% (Fig. 2). Complexes of hyaluronic acid and proteoglycan monomers not stabilized by link proteins are also stable in sucrose density gradients and they sediment as aggregates (Franzén *et al.*, 1981). Therefore the absence of any radioactive component sedimenting faster than the monomer at the short chase time suggests that the proteoglycan monomers and the hyaluronic acid are exported separately and are combined extracellularly.

In a similar experiment the medium, after 15min of chase, was incubated at 37°C in the absence of cells. After 24h of incubation the amount of radioactivity sedimenting as aggregates was 65%, showing that the presence of cells is not required for the assembly of the exported monomers into aggregates. The amount of radioactivity sedimenting as proteoglycan (aggregates plus monomers) was nearly identical at 15min of chase (12848c.p.m.) and 24h chase (14256c.p.m.). Thus 90% of the incorporated radioactivity recovered in the medium at 24h of chase is already secreted within 15min. The amount of free [$^{35}$S]sulphate left over from the pulse and recovered at the top of the sucrose density gradients constitutes about 45% (44.3 and 43.9% respectively) of the total radioactivity present in the medium, when the pulse–chase protocol described in the Experimental section is used.

*Size of aggregates*

The extracellular assembly of aggregates might proceed along two different lines. Each aggregate may be assembled separately and completed to achieve its final size before the next aggregate is formed. Alternatively, the different components of the aggregates, namely monomers, hyaluronic acid and link proteins, are combined at random, forming aggregates that gradually increase in size as the proportion of monomers bound to hyaluronic acid increases.

A pulse–chase experiment with a 15min pulse of [$^{35}$S]sulphate followed by 15, 30, 45, 90, 180 and 360min of chase was undertaken to discriminate between these two possibilities. The pulses were initiated at different times, so that the end of all chase times occurred simultaneously. Samples of each medium were immediately layered on sucrose density gradients prepared in advance. The sedimentation patterns of the aggregates were compared after 36min at 240000$g_{av.}$ (Fig. 3). By 15min of chase most of the label was recovered at the top of

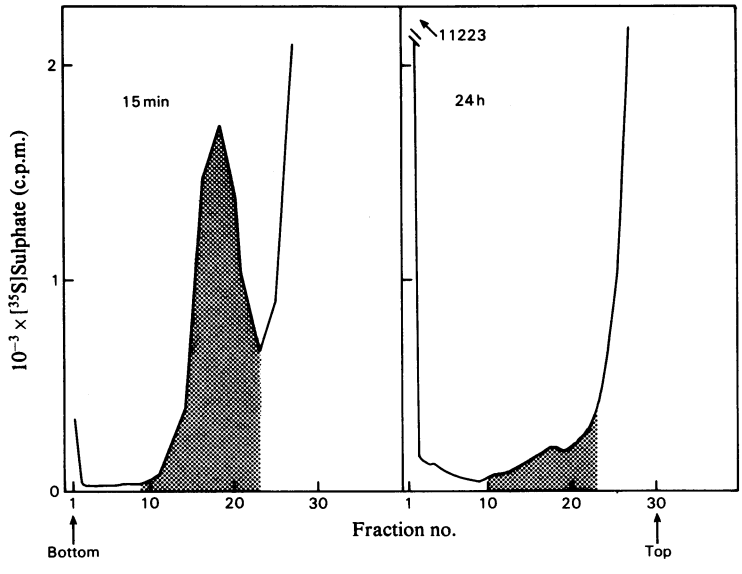


Fig. 2. *Proportions of proteoglycan aggregates in the medium from a pulse–chase experiment*
Experimental details are given in the text. The chase times are indicated in the Figure, which shows the distribution of [$^{35}$S]sulphate in sucrose density gradients centrifuged at 240000$g_{av.}$ for 3h. The stippled area indicates the position of the monomer.

the gradient at the position of monomers. A minor portion of the radioactivity, however, sedimented faster than the monomer peak, which suggests that some monomers (9%) had formed aggregates.

The proportion of the radioactivity sedimenting as aggregates increased with time (Figs. 3 and 7*a*). The rate of aggregation is highest at the beginning of the experiment (Fig. 7*a*). One explanation is that the monomers first bound to the hyaluronic acid exert a steric hindrance to the binding of other monomers to the same aggregate. The size of the aggregates also increased with time and can be measured as the proportion of radioactivity recovered in fractions 10–14 compared with the total radioactivity in fractions 2–23 of the sucrose density gradient. The size of the aggregates increased linearly for up to 3 h of chase (Fig. 7*a*) and was then approximately the same as for aggregates isolated from cultures incubated for 6 days (results not shown). With time more of the largest-size aggregates accumulated at the bottom of the tube (Fig. 7*a*). In a control experiment excess of monomer (3 mg/ml) was added

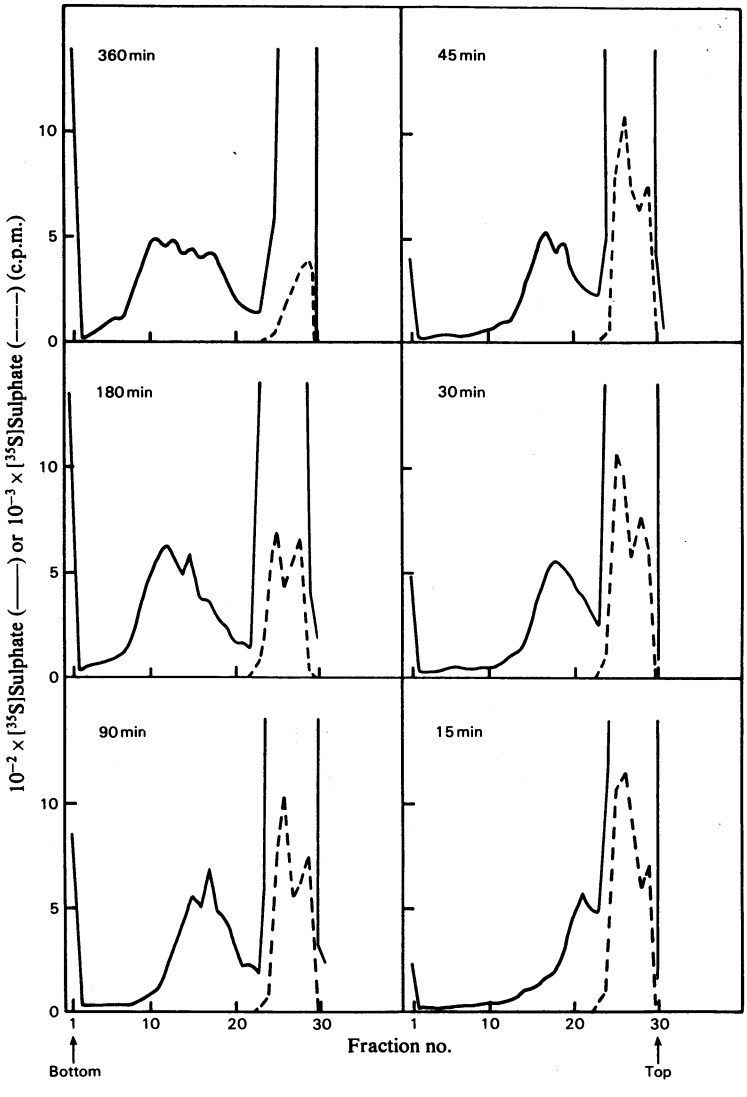


Fig. 3. *Size of proteoglycan aggregates in the medium from a pulse–chase experiment*
Experimental details are given in the text. The chase times are indicated in the Figure, which shows the distribution of [$^{35}$S]sulphate in sucrose density gradients centrifuged at 240000 $g_{av.}$ for 36 min.

to the cultures at the beginning of the chase. By 6 h of chase all radioactivity was still recovered at the top of the gradient (results not shown). The absence of any aggregates in the presence of excess of monomer in the medium suggests that the exogenous monomer takes part in the equilibrium with the endogenous hyaluronic acid and competes with the endogenous monomer for binding sites. The results indicate that the aggregation occurs at random in the medium, rather than as an ordered assembly of monomers to aggregates.

*Stability of the newly formed aggregates*

Aggregates, link-stable as well as link-free, remain stable during sedimentation in sucrose density gradients as discussed above. To discriminate between the two forms of aggregates, the following competition experiments were performed. A large excess of exogenous components was added to the medium in order to displace the monomers in link-free aggregates from the hyaluronic acid. Samples of the medium from the cultures that were pulsed with [$^{35}$S]sulphate for 15 min and then chased for 15, 30, 45, 90, 180 and 360 min were incubated for 24 h at 4°C with nasal proteoglycan monomers (3 mg/ml), hyaluronic acid oligosaccharide ($HA_{14}$ fraction; 0.2 mg/ml) or high-molecular-weight hyaluronic acid (0.2 mg/ml). Proteoglycan aggregates and monomers in the incubation mixtures were subsequently separated in sucrose density gradients centrifuged at 240000 $g_{av.}$ for 36 min.

*Incubation with exogenous monomer.* A major proportion of proteoglycan monomers will bind to added hyaluronic acid to form aggregates. Since the bound monomers are in equilibrium with free monomers, an exchange between free and bound molecules occurs continuously. Thus, if a large excess of exogenous monomers is added, most of the aggregated monomers will with time be derived from the exogenous monomer. Proteoglycan monomers in link-stable aggregates will not take part in the equilibrium with free monomer (Kimura *et al.*, 1979). Consequently the proportion of link-stable aggregates can be measured as aggregates remaining after incubation with a large excess of proteoglycan monomers. To assess the proportion of link-stable aggregates, samples of the medium from the pulse–chase experiment, discussed above, were incubated with nasal cartilage proteoglycan monomers for 24 h and analysed by centrifugation in sucrose density gradients (Figs. 4*a* and 4*b*; only the 15 min chase and 6 h chase are shown in the Figure). At no time did the exogenous monomer displace the endogenous monomer from the aggregates. Apparently, the endogenous monomers are bound in link-stable aggregates and do not take part in the equilibrium with free monomer. Furthermore, the exogenous monomer does not appear to compete with the endogenous monomer on equal terms, since the proportion of endogenous monomer bound to aggregates actually increased at short chase times (Fig. 7*b*).

At all times the size of the aggregates was so large that they sedimented to the bottom of the tube in 36 min. The increased size of the aggregates might be explained as binding of exogenous monomers to binding sites on the hyaluronic acid not occupied by endogenous monomers.

*Incubation with oligosaccharide ($HA_{14}$ fraction).* An $HA_{14}$ oligosaccharide can bind one proteoglycan monomer but is not sufficiently large to bind one monomer and one link protein (Hascall & Heinegård, 1974; Kimura *et al.*, 1979). If an excess of $HA_{14}$ fraction is added to a mixture of proteoglycan monomers and hyaluronic acid, the oligosaccharide will take part in the equilibrium. With time essentially all proteoglycan monomers will be bound to the $HA_{14}$ fraction. Link-stable proteoglycan aggregates will not take part in the equilibrium with the oligosaccharide.

Samples of the medium from the pulse–chase experiments were incubated with $HA_{14}$ fraction for 24 h and analysed by sucrose-density-gradient centrifugation (Figs. 4*c* and 4*d*). The proportion of aggregates increased at shorter chase times (Fig. 7*b*). This is the expected result if each monomer that becomes bound to the endogenous hyaluronic acid is immediately stabilized with link protein, whereas the monomer bound to the oligosaccharide still is in equilibrium with the population of free monomers. Consequently, an ever-increasing proportion of monomers will become part of link-stable aggregates, even in the presence of oligosaccharide (Kimura *et al.*, 1979). It is more obvious at short chase times, where the population of not-yet-aggregated monomers is larger. At later chase times the amount of proteoglycan aggregates remains constant, indicating that nearly all the aggregating monomers are already part of link-stable aggregates at the end of the chase. The aggregates sediment a shorter distance after incubation with oligosaccharide, indicating a smaller size compared with the analysis performed directly at the end of the chase.

*Incubation with high-molecular-weight hyaluronic acid.* Further tests for the presence of link-stable aggregates were done with excess of added high-molecular-weight hyaluronic acid. The exogenous hyaluronic acid will compete with endogenous hyaluronic acid for binding of free monomers. With time all monomers in link-free aggregates will become aggregated to the exogenous hyaluronic acid, with only a few monomers per molecule of hyaluronic acid. Since link-stable aggregates might be formed with exogenous high-molecular-weight hyaluronic acid but not with the oligosaccharide, the

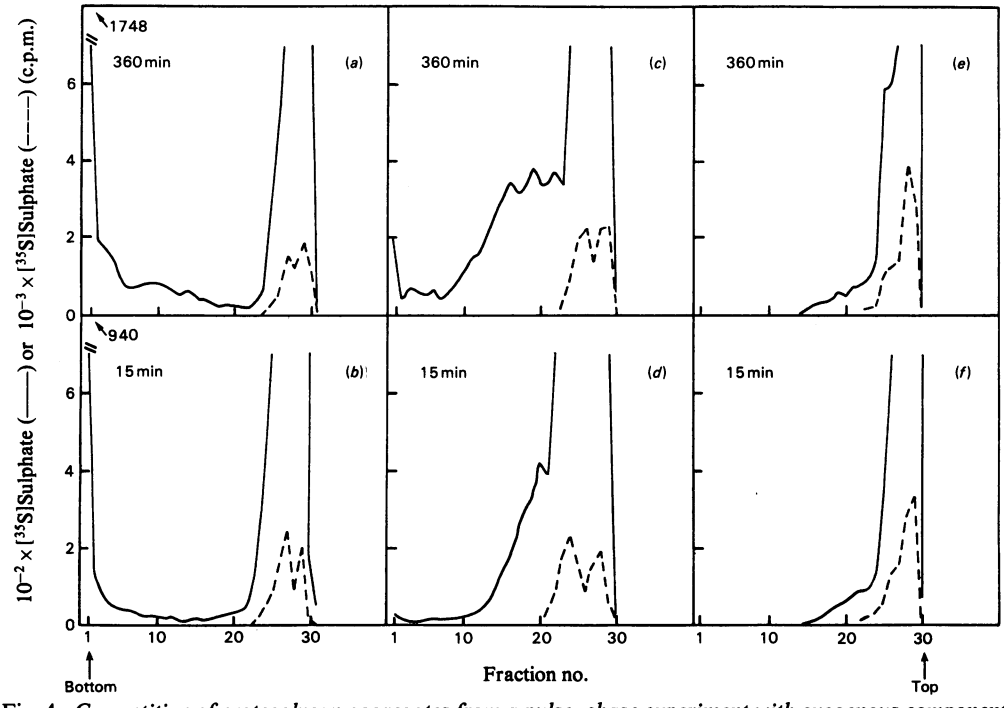


Fig. 4. *Competition of proteoglycan aggregates from a pulse–chase experiment with exogenous components*
Experimental details are given in the text. The Figure shows the distribution of [$^{35}$S]sulphate in sucrose density gradients centrifuged at 240000$g_{av.}$ for 36 min. The cultures were chased for the times indicated in the Figure, and the medium was incubated with (*a* and *b*) exogenous monomer, (*c* and *d*) hyaluronic acid oligosaccharides or (*e* and *f*) high-molecular-weight hyaluronic acid.

presence of hyaluronic acid in excess will inhibit further formation of large aggregates. In contrast, added oligosaccharides are not capable of binding monomer plus link proteins and will therefore only retard the formation of link-stable aggregates with the endogenous hyaluronic acid.

Samples of the medium from the pulse–chase experiment were incubated with hyaluronic acid for 24h and analysed by centrifugation in sucrose density gradients (Figs. 4*e* and 4*f*). Surprisingly, the exogenous hyaluronic acid not only competed for binding of free monomers with the endogenous hyaluronic acid, but also effectively displaced the already bound monomers at all chase times (Fig. 7*b*). Possibly at the very low concentration of proteoglycans in the chase medium there is a significant exchange between free and bound monomers even in the presence of link proteins. Accordingly, almost all proteoglycan monomers will become bound to the exogenous hyaluronic acid with time. In control experiments it has been shown that added high-molecular-weight hyaluronic acid does not affect the sedimentation of proteoglycan aggregates.

*Dissociation and reassociation*

Proteoglycans from cartilage are usually prepared by extraction of the tissue with 4M-guanidinium chloride, which dissociates the aggregates and allows the components to diffuse out of the tissue. During the subsequent dialysis to associative conditions the proteoglycan monomers, the hyaluronic acid and the link proteins reassociate. The procedure demonstrates the reversible denaturation of the proteins involved (Sajdera & Hascall, 1969). The proportion of monomers that reaggregate increases with increasing concentration (Franzén *et al.*, 1981). Experiments were performed to study the capacity of the newly synthesized monomers to denature reversibly and reassociate.

*Reassociation without exogenous proteoglycan.* One volume of 8M-guanidinium chloride was added to samples of the medium from the pulse–chase experiment to dissociate the aggregates. After dialysis to associative conditions, monomers and aggregates were separated by centrifugation in sucrose gradients (Figs. 5*a* and 5*b*). The re-

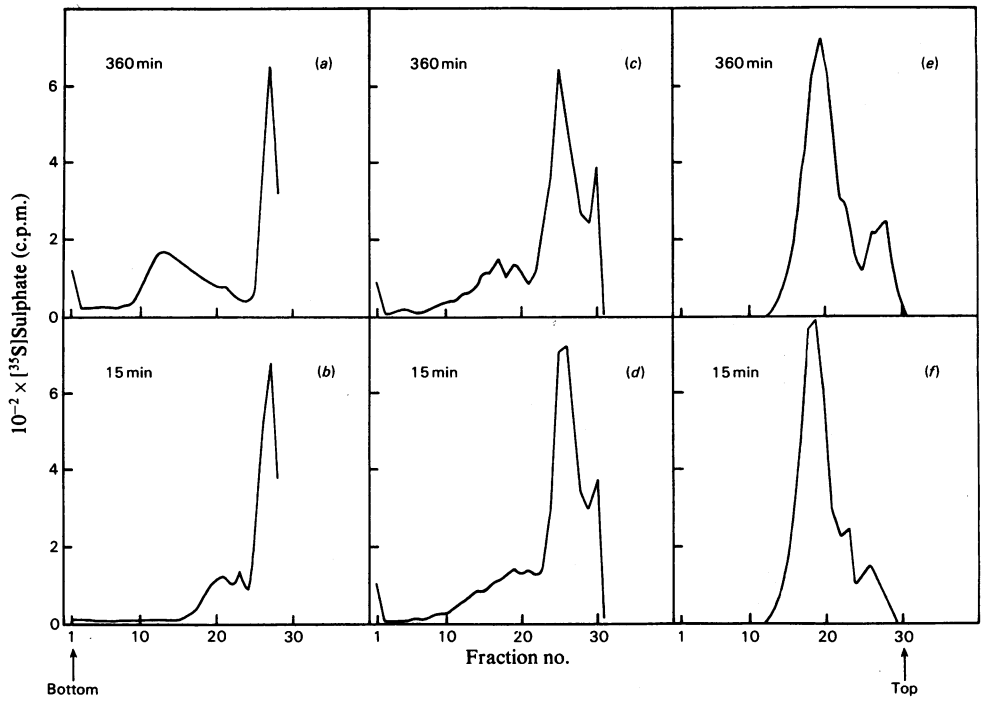


Fig. 5. *Dissociation–reassociation of proteoglycans from a pulse–chase experiment*

Experimental details are given in the text. The chase times are indicated in the Figure. (*a* and *b*) Reassociation without exogenous proteoglycan; the Figure shows the distribution of [$^{35}$S]sulphate in sucrose density gradients centrifuged at 240000$g_{av.}$ for 36 min. (*c* and *d*) Reassociation with exogenous aggregates; the Figure shows the distribution of [$^{35}$S]sulphate in sucrose density gradients centrifuged at 240000$g_{av.}$ for 1 h. (*e* and *f*) Reassociation with exogenous monomer; the Figure shows the distribution of [$^{35}$S]sulphate in sucrose density gradients centrifuged at 240000$g_{av.}$ for 3 h.

associations performed with only the low concentrations of proteoglycans present in the chase medium appear to proceed to near completion (Fig. 7*c*), although the actual values for aggregate content are somewhat scattered (Fig. 7*c*). The proportion and size of aggregates, however, were somewhat larger at long chase times compared with short times. This is not unexpected considering that unlabelled proteoglycans synthesized during the chase take part in the equilibrium.

*Reassociation with exogenous proteoglycan aggregates.* Proteoglycan A1 fraction (NC-A1 fraction) dissolved in 1 vol. of 8 M-guanidinium chloride was added to samples of the medium from the pulse–chase experiment. After dialysis, monomers and aggregates were separated by centrifugation in sucrose density gradients at 240000$g_{av.}$ for 1 h (Figs. 5*c* and 5*d*). Both the proportion and size of the reassociated aggregates were independent of the length of the preceding chase. Since the mixed aggregates formed were considerably smaller than the native aggregates formed in culture, the proteoglycan aggregates required 1 h of sedimentation for separation from monomers. In addition, the largest-size aggregates sedimenting to the bottom of the tube on direct analysis were not present. The proportion of aggregate was constant, indicating that in the presence of exogenous aggregates the reassociation proceeds reproducibly. Since the endogenous proteoglycans have equal capacity to form mixed aggregates regardless of time spent in culture, extracellular processing resulting in improved aggregatability is unlikely. The proportion of proteoglycan monomers that reaggregate is concentration-dependent, and at the 3 mg/ml used in this experiment only half of the exogenous proteoglycan is reaggregated (Franzén *et al.*, 1981); compare also with Fig. 6*c* below). Proteoglycan A1 fraction contains a proportion of aggregating proteoglycan monomers not bound into aggregates (Heinegård & Hascall, 1979). The proportion of the endogenous monomer recovered in the reassociated mixed-aggregate fraction (Fig. 7*c*) is lower than that in the native aggregates (Fig. 7*a*). Whether the endogenous monomer is less efficiently renatured during reassociation or the excess of exogenous proteo-

glycan interferes with the binding of link to the endogenous monomer is not known. Since nearly all link proteins present are of exogenous origin, it is likely that the endogenous monomer can interact with the exogenous link protein. Otherwise the excess of free exogenous monomer would displace all endogenous link-free monomers. However, the proportion of endogenous monomers aggregated is significantly lower when reaggregated with exogenous aggregates than in the medium after 6h of chase.

*Reassociation with exogenous monomer.* Proteoglycan monomers (A1-D1 fraction) were dissolved in 8M-guanidinium chloride and 1vol. of the solution was added to samples of the medium from the pulse–chase experiment. After dialysis, the sedimentation in sucrose density gradients was performed at 240000$g_{av.}$ for 3h. When reassociation is performed in the presence of a large excess of proteoglycan monomer, the exogenous monomer will compete for the binding sites on the hyaluronic acid and only a negligible portion of the endogenous monomer will become bound. As expected, at all chase times the radioactivity sedimented to the position of free proteoglycan monomers (Figs. 5*e* and 5*f*). If the endogenous link protein were able to bind only to the endogenous monomer, however, these molecules should with time become irreversibly bound to the hyaluronic acid, even in the presence of excess of exogenous monomer. Therefore the absence of any radioactive aggregates (Fig. 7*c*) indicates that the endogenous link protein is capable of binding also to the exogenous nasal cartilage proteoglycan monomers. The sedimentation of endogenous monomers shows no variation with time spent in culture, indicating that degradation does not occur during the chase period.

*pH-stability of proteoglycan aggregates.* Previous work (Tang *et al.*, 1979) has shown that link-stable and link-free proteoglycan aggregates differ with regard to stability at low pH. Thus link-free aggregates are dissociated at pH4.0, whereas link-stabilized aggregates are stable. These results were verified by using sucrose-density-gradient centrifugation to separate proteoglycan monomers and aggregates. A mixture of proteoglycan monomer (A1-D1 fraction) and hyaluronic acid was incubated at pH4.0 for 24h, and the proportion of aggregates was determined by sucrose-density-gradient centrifugation (Fig. 6*a*). Very little absorbance was detected at the position of aggregates. In contrast, proteoglycan A1 fraction incubated at pH4.0 showed the same sedimentation profile as was obtained at pH7.0 (results not shown). Samples of the medium from the pulse–chase experiment were adjusted to pH4.0 and analysed by sucrose-density-gradient centrifugation. No aggregates were detected at any time (Fig. 6*b*). It is likely, then, that

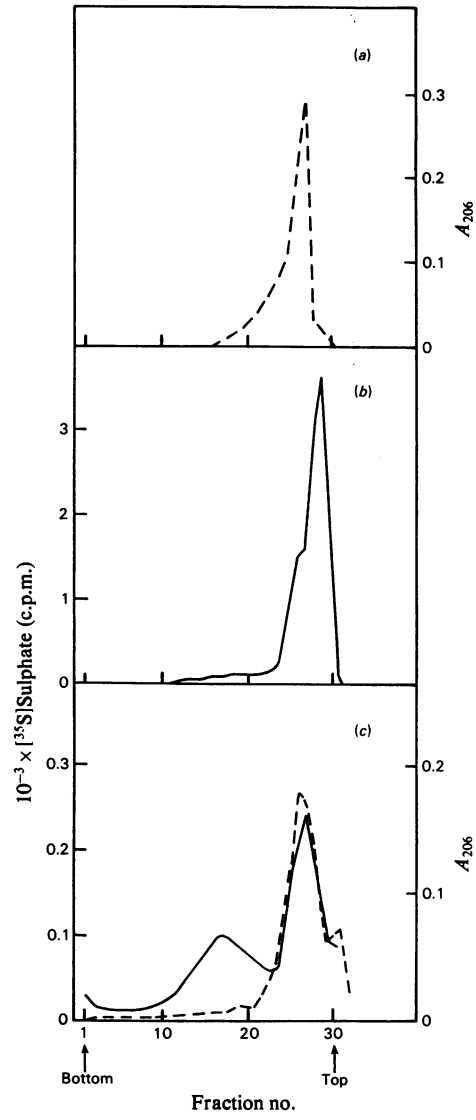


Fig. 6. *pH-stability of proteoglycan aggregates prepared from different sources*

Experimental details are given in the text. The Figure shows the distribution of [$^{35}$S]sulphate (——) or absorbance at 206nm (– – – –) in sucrose density gradients centrifuged at 240000$g_{av.}$ for 36min. (*a*) NC-A1-D1 fraction (3mg/ml) and hyaluronic acid (0.03mg/ml) incubated at pH4.0 for 24h. (*b*) Medium from cultures pulsed with [$^{35}$S]sulphate and chased for 6h and then incubated at pH4.0 for 24h. (*c*) Medium from cultures pulsed with [$^{35}$S]sulphate and chased for 6h and then dissociated–reassociated with exogenous aggregates as in Figs. 5(*c*) and 5(*d*). The reassociated sample was incubated for 24h at pH4.0 before analysis.

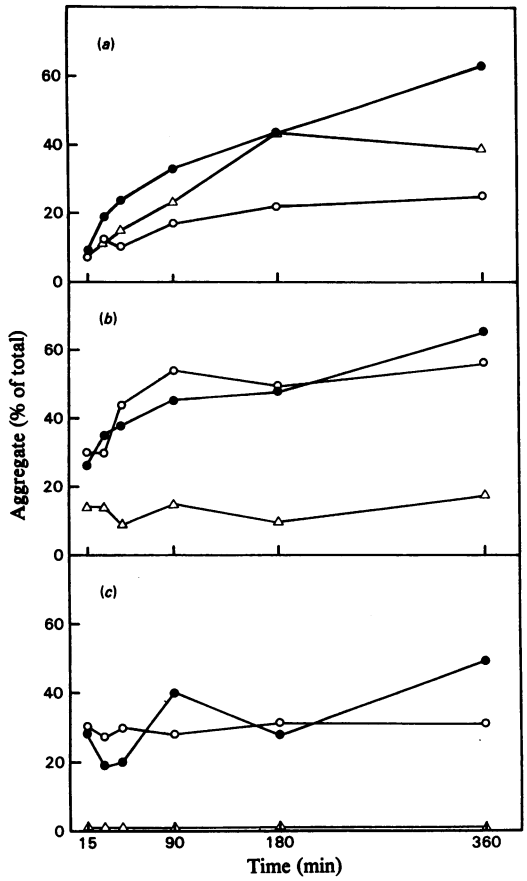


Fig. 7. *Proportion of proteoglycan aggregates in medium from a pulse–chase experiment*

The chase times are indicated in the Figure. (*a*) ●, Medium analysed immediately at the end of the chase; ○, proportion of aggregates recovered in the bottom fraction of aggregates in fraction 2–23; △, proportion of aggregates recovered in fraction 10–14 of aggregates in fraction 2–23. (The results are obtained from the experiment shown in Fig. 3.) (*b*) ●, Medium analysed after incubation with excess of nasal-cartilage monomer; ○, medium analysed after incubation with excess of hyaluronic acid oligosaccharide; △, medium analysed after incubation with excess of high-molecular-weight hyaluronic acid. (The results are obtained from the experiments shown in Fig. 4.) (*c*) ●, Medium analysed after dissociation–reassociation without exogenous proteoglycan; ○, medium analysed after dissociation–reassociation with exogenous aggregates; △, medium analysed after dissociation–reassociation with exogenous monomers. (The results are obtained from the experiment shown in Fig. 5.)

the aggregates assembled in culture are different from those isolated from the tissue. The link protein may either be synthesized in a form that is less stable at low pH, or the hyaluronic acid-binding region of the monomer may not be able to bind the hyaluronic acid or the link protein in the same way as the tissue-derived monomer. In order to test these two possibilities, proteoglycan A1 fraction in 8M-guanidinium chloride was added to equal portions of the medium from the pulse–chase experiment. The dissociated proteoglycans were reaggregated by dialysis to associative conditions. After dialysis, the pH was adjusted to 4.0 and the solutions were incubated for 24h. Aggregates and monomers were separated in sucrose density gradients. The position of the exogenous proteoglycan was monitored as absorbance at 206nm and the position of the endogenous proteoglycan as radioactivity (Fig. 6*c*). The absorbance tracing is typical of a nasal-cartilage aggregate preparation, whereas the radioactivity sedimented as free proteoglycan monomer. Provided that the endogenous and the exogenous link proteins compete on equal terms for binding to the endogenous monomer, most of the endogenous monomers are stabilized by the large excess of exogenous link protein. Thus the result suggests that the low stability of endogenous aggregates at low pH is due to differences in the hyaluronic acid-binding region of the culture proteoglycan rather than to an altered link protein.

## General discussion

Sedimentation in sucrose density gradients gives a very rapid separation between monomers and aggregates and can be performed without added carrier. Thus the presence of only free proteoglycan monomer and no aggregates at short times reported in the present paper indicates that the aggregation with hyaluronic acid is an extracellular phenomenon. Separate pathways of synthesis and export are then required, since even the extracellular proteoglycan monomers are able to interact with hyaluronic acid added with nasal-cartilage proteoglycan aggregates to 4M-guanidinium chloride extracts of the cells.

The present investigation corroborates and extends data by Kimura *et al.* (1979). It is shown directly that formation of hyaluronic acid–proteoglycan aggregates is an intracellular event and that the aggregates formed became rapidly stabilized by link protein. Kimura *et al.* (1979) used competition with proteoglycan monomer to show that formation of link-protein-stabilized aggregates is an extracellular event. The use of suspension cultures, however, provides an advantage, in that 90% of the proteoglycans can be accounted for in the medium already at short chase times compared with about 50% for the proteoglycans from the monolayer culture.

The sedimentation rate of proteoglycan aggregates in a sucrose density gradient depends on the number of monomers bound per molecule of hyaluronic acid (Franzén *et al.*, 1981*a*). Thus the

sedimentation patterns show that the size of the aggregates is smaller, and the proportion of bound monomers is lower, at short chase times than at long chase times. At longer chase times a number of aggregates are large enough to sediment to the bottom at 36 min. There is not, however, a continuous distribution of the aggregates in the sucrose density gradient. Rather, the aggregates sedimenting to the bottom of the tube may represent a population distinct from the major population of aggregates. A level of organization above the proteoglycan monomer–hyaluronic acid aggregates, the so-called superaggregates, has been postulated (Pita *et al.*, 1975).

The newly formed aggregates were stable to competition with hyaluronic acid oligosaccharides but not stable to competition with hyaluronic acid. This result might be explained if the free monomers were present mainly as monomer–link complexes, as suggested by Kimura *et al.* (1980). An oligosaccharide cannot bind both the monomer and the link protein, whereas the hyaluronic acid can. The monomer–link complex assumingly has a lower affinity for the oligosaccharide than for the hyaluronic acid. In the presence of exogenous oligosaccharide, the monomer without link will bind to the oligosaccharide, whereas the monomer–link complex will bind preferentially to the endogenous hyaluronic acid. In the presence of exogenous hyaluronic acid, however, any free monomer–link complex, as well as the monomer without link, will bind to the exogenous hyaluronic acid.

The proportion of aggregates present at short chase times increased even on incubation with exogenous monomer, although not to the high value of 65% aggregate obtained when medium alone was incubated for 24 h in the absence of cells. This result supports the presence of monomer–link complexes as discussed above, since the exogenous monomer does not compete with the free endogenous monomer on equal terms. It appears, then, that at least a proportion of the endogenous monomers are present as monomer–link complexes and that some of these complexes bind to hyaluronic acid before the exogenous monomer competes for binding of the link protein. In contrast, no aggregates were formed when exogenous monomer was present during the chase, indicating that the exogenous monomer competed for the newly synthesized link protein at the time of export. In conclusion, the hyaluronic acid molecules, proteoglycan monomers and the link proteins are exported separately and combined in the medium so that monomer–link complexes are formed, which then bind to the hyaluronic acid to form the link-stable aggregates.

In a previous study the proteoglycans isolated by CsCl-density-gradient centrifugation from the medium of chondrocyte cultures were characterized (Björnsson & Heinegård, 1981*b*). The presence of link proteins in such preparations was demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Trypsin digestion of link-stable aggregates prepared from cartilage has been used to isolate the hyaluronic acid-binding region of the proteoglycan protein core attached to the hyaluronic acid and protected by the link protein (Heinegård & Hascall, 1974). The complex is resistant even to limit digestion with the enzyme (Heinegård & Axelsson, 1977). If not protected by link proteins, however, the hyaluronic acid-binding fragment is still not appreciably degraded, but its function is lost (Heinegård & Hascall, 1979). In contrast, the proteoglycan isolated from the culture medium was not resistant to trypsin, and was degraded to small peptides by limit digestion with trypsin regardless of whether in link-stabilized aggregate or monomer form (Björnsson & Heinegård, 1981*b*). The differences between the medium proteoglycan and the nasal cartilage proteoglycan are also demonstrated by the different stabilities of the aggregates at low pH. At pH 4.0 all the endogenous monomers were displaced from mixed aggregates, whereas most of the exogenous monomer remained aggregated. These results might be interpreted as a relatively low affinity of the hyaluronic acid-binding region of the endogenous proteoglycan monomer either for the link protein or for the hyaluronic acid. Thus the lower stability of the proteoglycan aggregates synthesized *in vitro* encountered might result from a difference in the conformation of the hyaluronic acid-binding region. Whether the lower stability of the proteoglycan aggregates from the culture medium represents an earlier state of maturation or an artifact derived from the conditions used *in vitro* is not known.

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