The effect of age on the incorporation of newly synthesized aggrecan into the extracellular matrix of human articular cartilage was investigated. This property was measured in a pulse-chase explant culture system by determining the distribution of radiolabeled molecules ([35S]sulfate-labeled) between a nondissociating extract (phosphate-buffered saline), which extracts mainly nonaggregated macromolecules, and a dissociating extract (4 M GdnHCl) containing mainly aggrecan that was complexed in situ with hyaluronan. The rate of incorporation of aggrecan into aggregates was much slower in mature cartilage than in tissue obtained from younger individuals. Furthermore, autoradiography showed that in mature cartilage, newly synthesized aggrecan is not transported from the pericellular environment within the first 18 h of chase culture, whereas in immature cartilage, it moves into the intercellular space during the same period, i.e. aggrecan is processed in the extracellular space very differently in young and adult articular cartilage. Experiments were also performed to show that the interaction of link protein with newly synthesized aggrecan depends on the maturity of the G1 domain of aggrecan. This investigation has shown that the extracellular aggregation of aggrecan in adult human articular cartilage involves a number of intermediate structures. These have not been identified in the very young cartilage obtained from laboratory animals or in porcine and bovine articular cartilage obtained from the abattoir.

Aggrecan is one of the major macromolecules in the extracellular matrix of articular cartilage and endows the tissue with its characteristic water imbibing properties and its ability to undergo reversible compression. During normal aging of cartilage, aggrecan undergoes many posttranslational modifications to its protein core and to the number, size, and proportion of the chondroitin sulfate and keratan sulfate chains that are covalently associated with it (1). These events are mostly catabolic in nature and are a consequence of the relatively long resident time of aggrecan in the matrix (2). However, it is clear that a number of these age-related changes could only have come about by an altered anabolic response of the chondrocyte (3).

A recent publication (3) has emphasized that the rate of aggrecan synthesis (sulfate incorporation) is dependent on the age of the specimen from which the tissue was obtained. However, the ability of the chondrocyte to make this molecule is only one part of the maintenance process; it also has to be assembled into aggregates in the extracellular matrix. This process involves the interaction of aggrecan with link protein(s) and hyaluronan, which are themselves under their own biosynthetic control, and the complex must then be deposited within the correct compartment of the tissue (4). Thus, extracellular assembly must be a rate-limiting step in the formation of a stable matrix. The speed at which aggregation happens is important for normal tissue homeostasis, but it would be particularly important during any pathological process when the chondrocyte attempts to repair a defective extracellular matrix.

The study by Oegema (5) was the first to indicate that aggrecan was not immediately incorporated into aggregates when it was exported from the cell. Subsequent studies also showed that the disulfide bonds present in the G1 domain of aggrecan required time to mature and that aggrecan is only slowly converted from a form that has a low affinity for hyaluronan to one with a higher affinity (6–12). The latter investigations also showed that the presence or absence of link protein influenced the rate of incorporation of aggrecan into aggregates. Moreover, a study of “delayed aggregation” in rabbit articular cartilage showed that it also operates in vivo and that the phenomenon is age-dependent (13). A more recent study demonstrated that the extent of tissue compression, with the concomitant changes in proteoglycan concentration and pH of the extracellular matrix, could influence the hyaluronan binding properties of aggrecan in bovine cartilage explants (14). This lead the authors to suggest that such a pH mechanism may have a physiological role, promoting proteoglycan deposition in regions of low proteoglycan concentration. Thus, at least one biological mechanism is active in articular cartilage that has the ability to control the rate of aggrecan assembly.

There have been no direct measurements of the rate of aggregation of aggrecan in articular cartilage and no investigations of how normal aging affects the process. The experiments presented here support a hypothesis in which the age of the individual is a very important factor in controlling the rate and extent of aggregation in human articular cartilage.

**EXPERIMENTAL PROCEDURES**

**Tissue Source**—Intact knee joints were obtained from amputations or massive replacements at operations for bone tumors not involving the joint space. Fresh specimens (within 1 h of operation) were dissected using sterile techniques, and full-thickness cartilage was removed from all of the femoral condyle and diced into 1–2 mm² pieces. The tissue was thoroughly mixed to ensure that representative samples could be taken.

**Tissue Culture**—The exact conditions for explant culture depended on the analysis that was required. The rate of proteoglycan synthesis was determined, by measuring the incorporation radioisotope into articular cartilage; triplicate aliquots (~50 mg) of tissue were incubated for 4 h in 1 ml of Dulbecco’s modified Eagle’s medium containing 20 μCi...
of $[^{35}S]$sulfate, which labeled glycosaminoglycan chains associated with aggrecan. A quantitative measure of aggrecan biosynthesis was calculated as nmol/h/g of wet weight of cartilage, as described by Bayliss et al. (3). When pulse-chase experiments were carried out in order to determine the rate of aggregation, glycosaminoglycan chains associated with aggrecan were radiolabeled by incubating the tissue for 1 or 4 h in Ham’s F-12 culture medium containing 150 μCi/ml $[^{35}S]$sulfate, i.e. in culture medium containing 15–30 μM $[^{35}S]$SO$_4 ^{2–}$. This produced a pool of newly synthesized aggrecan, in situ, that was of a high specific activity. After storing an aliquot (−200 mg) at −20 °C, the remainder of the cartilage was incubated in Dulbecco’s modified Eagle’s medium, i.e. in culture medium containing 800 μM $[^{35}S]$SO$_4 ^{2–}$. After various periods of time in chase culture, cartilage was removed and stored at −20 °C until it was required. When long term, 4-h pulse-chase experiments were performed, the chase culture medium was also retained and stored at −20 °C. Preliminary experiments compared the structure of aggrecan synthesized by cartilage cultured in Ham’s F-12 medium or Dulbecco’s modified Eagle’s medium (results not shown). No differences in the hydrodynamic size or the electrophoretic profile of aggrecan or in the Δdi6S:Δdi4S ratio of the chondroitin sulfate chains were observed during the short culture period used.

**Extraction of $[^{35}S]$-Labeled Proteoglycans—**Cartilage samples were sectioned at 20 μm in order to overcome any barrier to diffusion and to ensure complete extraction of the aggregate components, before extracting them in PBS (4 ml) overnight at 4 °C. The tissue sections were filtered through glass wool and then extracted for a further 24 h at 4 °C with 4 M GnHCl (4 ml). Both extracts were stored at −20 °C until they were analyzed, and the cartilage residue was washed with PBS and dispersed by digesting it with papain. The distribution of radiolabel ($[^{35}S]$sulfate) in each extraction solution and in the papain digest was determined by scintillation counting. The proportion of radiolabeled macromolecules released into the culture medium of the long term chase cultures was determined by measuring the $[^{35}S]$sulfate excluded from PD10 columns (Amersham Pharmacia Biotech). All extraction buffers contained proteinase inhibitors (7).

**Gel Chromatography—**Size exclusion gel chromatography was performed on a column (150 × 0.65 cm) of Sepharose CL2B. Cartilage extracts (1 ml) were eluted either with 0.5 M sodium acetate, pH 7.4, or with 2 M GnHCl buffered with 50 mM sodium acetate, pH 7.4. Fractions were analyzed for radioactivity by scintillation counting and for their uronic acid content (15).

**Agarose/Gel Electrophoresis—**Agarose/polyacrylamide gels were prepared as described in Refs. 16 and 3. The gels were used to assess the aggregating properties of newly synthesized aggrecan, in which case the polyacrylamide gels were equilibrated in 0.01 M Tris acetate, pH 7.4 (associating conditions). When it was necessary to assess the electrophoretic profile of aggrecan itself, the gels were equilibrated in 4 M urea, and the samples were buffer-exchanged into 7 μM urea (dissociating conditions). Endogenous proteoglycans (3 μg of uronic acid/well) were detected by staining the gel with toluidine blue, and radioactive bands were visualized by drying the stained gel on Gel Bond and subjecting it to autoradiography using Eastman Kodak Co. X-Omat film. The electrophoretic conditions used were those described by Bayliss et al. (3).

**Autoradiography—**The distribution of $[^{35}S]$sulfate within the tissue was determined by autoradiography of immature and mature cartilage that had been subjected to the 4-h pulse and 18-h chase protocol described above. After culture, tissue samples were fixed in 10% (v/v) formaldehyde/saline and embedded in wax before cutting 7-μm histological sections and collecting them on glass slides. Slides were coated with Kodak K5 silver emulsion and stored in the dark for 4 weeks. The slides were then developed and mounted and the distribution of silver grains visualized by light microscopy using a Leitz microscope. Less than 1% of the incorporated $[^{35}S]$sulfate was released into the culture medium during the period of incubation. Although no analysis was made of the tissues after fixation, embedding, and sectioning, cysteine and iodoindium chloride was included in the fixative to prevent leaching of the proteoglycans during processing (17).

**Preparation of Hyaluronan Oligosaccharides—**Hyaluronan oligosaccharides were prepared by digesting a solution (1 mg/ml) of human umbilical cord hyaluronan (Sigma) with ovine testicular hyaluronidase (Worthington Biochemicals, Freehold, NJ) at a concentration of 40 μg/ml in 0.2 M sodium acetate containing 0.1 M NaCl at 37 °C for 4 h. The reaction was stopped by boiling the digest for 5 min, and the resultant precipitate was isolated by centrifugation. The supernatant, containing hyaluronan digestion products, was tested for its ability to compete with full-length hyaluronan chains for the binding of porcine aggrecan. Porcine aggrecan (1 mg/ml A1D1) was mixed with 5% (w/w) hyaluronan overnight at 4 °C, and the extent of aggregation was assessed by gel exclusion chromatography on Sepharose CL2B (results not shown). A 10-fold excess of the hyaluronidase supernatant, relative to the full-length hyaluronan concentration, was added to the mixture of hyaluronan and porcine A1D1. This procedure inhibited the formation of aggregates and disrupted preformed complexes formed between aggrecan and hyaluronan (results not shown).

**Preparation of Link Protein—**Link protein was prepared from a porcine aggregate (A1) preparation as described in Refs. 18 and 19.

**RESULTS**

**Age-related Changes in the Structure of Newly Synthesized Aggrecan—**Preliminary experiments compared the size exclusion profiles of $[^{35}S]$-labeled aggrecan, in direct 4 M GnHCl extracts of immature and mature human articular cartilage. Fig. 1 shows that although the endogenous aggrecan (uronic acid profile) is reduced in molecular size during aging and that there is an increase in molecular heterogeneity, the newly synthesized aggrecan ($[^{35}S]$-labeled) has the same $K_{av}$ in immature and mature cartilage. Electrophoresis in agarose/polyacrylamide gels emphasized this age-related change in aggrecan structure. The relationship between the three molecular species identified in adult cartilage extracts was investigated by radiolabeling aggrecan during a 4-h pulse with $[^{35}S]$sulfate and then chasing the culture in nonradioactive medium for 14 days. After electrophoresis of the 4 M GnHCl extracts in dissociative (8 M urea) composite gels, a comparison of the toluidine blue stained gel indicated the presence of three aggrecan bands in the pulse and the chase explants; these appeared to be of equal intensity, although no quantitative analysis was carried out (Fig. 2a). Fluorography indicated that most of the newly synthesized aggrecan was associated with the slower migrating, higher molecular weight species, identified by toluidine blue staining (Fig. 2b). However, a small proportion of the $[^{35}S]$-labeled aggrecan was also associated with the faster migrating bands, even in the extract of cartilage that had only been pulse-labeled for 4 h.

**Short Term, Pulse-Chase Protocol and Extraction Procedure to Measure the Rate of Aggregate Assembly—**In order to determine whether there were any age-related differences in the
extracellular assembly of aggrecan, cartilage explants were pulse-labeled for 1 h with $^{35}$S sulfate, and a portion of the tissue was stored at $-20 \, ^\circ\text{C}$. The remainder of the cartilage was cultured (chased) for up to 7 h in nonradioactive medium (Dulbecco's modified Eagle's medium), and samples of cartilage were removed every hour and stored at $-20 \, ^\circ\text{C}$ before processing them. In some cases, cartilage explants were chased for 48 h before terminating the cultures. All cartilage samples were cryosectioned at 20 $\mu$m prior to extraction with PBS (this extracts aggrecan that is presumed not to be complexed with hyaluronan) and then with 4 M GnHCl (this procedure removes aggrecan presumed to be complexed with hyaluronan). Fig. 3, a–i, shows that in immature cartilage, newly synthesized aggrecan is rapidly incorporated into the 4 M GnHCl-extractable pool and that with increasing age, the $^{35}$S sulfate-labeled aggrecan is retained for a longer period of time as PBS-extractable molecules.

**Long Term Pulse-Chase Cultures of Immature and Mature Cartilage Explants**—The slower rate of incorporation of aggrecan into aggregate complexes in adult cartilage meant that it was necessary to perform additional experiments in order to fully assess this process. Explants were, therefore, radiolabeled for 4 h before subjecting them to chase culture for up to 15 days. Once again, in immature cartilage, aggrecan was incorporated relatively rapidly into the 4 M GnHCl-extractable pool and that with increasing age, the $^{35}$S sulfate-labeled aggrecan is retained for a longer period of time as PBS-extractable molecules.

**Autoradiography of Radiolabeled Cartilage**—The quantitative results described above suggested that the rate of incorporation of aggrecan into aggregates was much slower in adult cartilage. In order to determine whether this meant that the newly synthesized molecules were present in different compartments of the tissue at these times, the distribution of radiolabeled aggrecan was visualized in situ by autoradiography, after a 4-h pulse and 4-h pulse-18-h chase culture of cartilage explants. After radiolabeling with $^{35}$S sulfate for 4 h, proteoglycans were confined to the pericellular compartment of both immature and mature chondrocytes. By the end of the 18-h chase period, however, most of the labeled aggrecan was localized in the intercellular matrix of immature cartilage. In marked contrast, the majority of newly synthesized molecules remained in the pericellular compartment of adult tissue during the first 18 h of chase culture (Fig. 4).

**Analysis of Aggrecan-Hyaluronan-Link Protein Interactions in Cartilage Extracts Using Gel Exclusion Chromatography**—The organization of aggrecan in situ was investigated further by studying the structure of the molecules extracted from the tissue. This was initially carried out using size exclusion chromatography. Fig. 5 illustrates the Sepharose CL2B profiles of extracts from a 9-year-old's and a 65-year-old's specimen of human articular cartilage. When the nondissociative (PBS) and dissociative (4 M GnHCl) extracts were combined in the relevant proportions (v/v) and then dialyzed to remove the guanidinium hydrochloride, 66% of the newly synthesized aggrecan extracted from the 9-year-old's cartilage (1 h pulse) was excluded from the column, indicating that this proportion of the extract could form aggregates. This value did not change during the subsequent 24-h chase period. In marked contrast, only 31% of the combined extracts from the 65-year-old's cartilage (4-h pulse) was excluded from Sepharose CL2B, and this proportion continued to increase during the following 3 days of chase culture, up to a value of 63%, i.e. aggrecan from adult cartilage exhibited “delayed aggregation.” The percentage of uronic acid (as a measure of the endogenous molecules) (Fig. 5,
Vo) was unchanged during the culture of both immature and mature tissue and remained at approximately 65% (results not shown).

Although 63% of the radiolabeled aggrecan could be extracted from immature cartilage with PBS (2 years; Fig. 3), the majority of newly synthesized aggrecan in this extract (62%) was recovered in the void volume of the column as aggregate (Fig. 6). However, nearly all of the 35S-labeled aggrecan in the PBS extract of adult cartilage (80 years) eluted in the included fractions of the column and this proportion changed very little during the subsequent 10 days of chase culture (Fig. 7). It should also be noted that the aggregation of the porcine aggrecan, added to these cultures to ensure that the conditions for assembly were kept constant, was not affected by the chase conditions, and approximately 70% of the uronic acid was eluted from the Sepharose CL2B column in each case.

Addition of exogenous hyaluronan (up to 4% (w/w) of the
subjected to size exclusion gel chromatography in order to as-

pulse-3-day chase explants from an 83-year-old individual were

ing, and the percentage excluded from the column was calculated.

addition of oligosaccharides of hyaluronan. Addition of link

extract of the pulse culture to complex with hyaluronan, but

casted more of the newly synthesized aggrecan in the PBS

again observed. The addition of link protein to the mixture

aggregates, and the phenomenon of delayed aggregation was

after a 4-h pulse and also after 3 days in chase culture, was not

sized aggrecan extracted with PBS from this adult specimen,

of adult aggrecan. Fig. 8 shows that most of the newly synthe-

tion of exogenous link protein could influence the aggregation

rate-limiting factor in the formation of aggregates. Therefore,

possible that the concentration of link protein may have been a

rides of hyaluronan (results not shown). It was considered

uronic acid content of the extract) to the PBS extracts of adult
cartilage demonstrated that the aggrecan in these extracts

exhibited the classical signs of delayed aggregation; they slowly

acquired the ability to interact with lower concentrations of

hyaluronan (Fig. 7). However, even when aggrecan was deemed

to have undergone the structural changes necessary for con-

version of a low affinity to a high affinity form, it was still

possible to extract it in a nonaggregated form with PBS. Fur-

thermore, a large proportion of the complex that formed with

hyaluronan was open to competition with high concentrations

of oligosaccharides of hyaluronan, i.e. the aggrecan that is

extracted with PBS at all culture times does not form link-

stabilized complexes with any endogenous link protein in the

extract when it is mixed with hyaluronan (results not shown).

This contrasts with the investigations of the aggregating prop-

erties of radiolabeled aggrecan in the PBS extracts of immature

cartilage, which were excluded from Sepharose CL2B columns,

and this profile was unchanged in the presence of oligosaccha-

rides of hyaluronan (results not shown). It was considered

possible that the concentration of link protein may have been a

rate-limiting factor in the formation of aggregates. Therefore,

experiments were carried out to determine whether the addi-

tion of exogenous link protein could influence the aggregation

of adult aggrecan. Fig. 8 shows that most of the newly synthe-

sized aggrecan extracted with PBS from this adult specimen,

after a 4-h pulse and also after 3 days in chase culture, was not

aggregated. Addition of hyaluronan induced the formation of

aggregates, and the phenomenon of delayed aggregation was

again observed. The addition of link protein to the mixture

caused more of the newly synthesized aggrecan in the PBS

extract of the pulse culture to complex with hyaluronan, but

these complexes were not stable and could be dissociated by the

addition of oligosaccharides of hyaluronan. Addition of link

protein had little effect on the extent of aggregation of the

chase cultures.

Analysis of Aggrecan-Hyaluronan-Link Protein Interactions

in Cartilage Extracts Using Agarose/Polyacrylamide Gel Elec-

trophoresis—The complexity of the aggregation process illus-

trated by the Sepharose CL2B studies indicated that there was

a need to confirm the findings using another method of inves-

Thus, the acquisition of aggregating potential and stability and

the role of link protein on this process was also investigated

using composite gel electrophoresis. The PBS and

4 m GdnHCl extracts from a 14-year-old’s and a 47-year-old’s

specimen were combined as described above and after electrop-

horesis in associative (Tris-buffered) composite gels, the ex-

tent of aggregation of the newly synthesized aggrecan was

assessed by fluorography. Fig. 9a shows that although most of

the newly synthesized aggrecan in the 4-h pulse extract of the

14-year-old’s specimen electrophoresed within the gel, a small

amount was retarded as higher molecular weight material.

Although this higher molecular weight complex persisted in

the extracts of immature cartilage explants that were chased

for 1, 2, 3 days, most of the [35S]-labeled aggrecan was recovered

as very high molecular weight complexes that did not enter the

gel. Furthermore, although some aggrecan was dissociated

from the high molecular weight complexes by oligosaccharides

of hyaluronan (Fig. 9b), the presence of exogenous link protein

stabilized these to the electrophoretic field so that they did not

then enter the gel (Fig. 9c). In marked contrast, almost all of

the radiolabeled aggrecan in the 4-h pulse extract of adult

cartilage migrated within the composite gel, indicating that it

could not interact with hyaluronan (Fig. 9d). During chase

culture, some of the aggrecan was retarded within the gel,

migrating as higher molecular weight complexes, but no radi-
labeled aggrecan was completely excluded from the gel. All of

the aggregates formed by aggrecan in the extracts of chase

explants of mature cartilage were unstable in the presence of

oligosaccharides of hyaluronan, whereas, when link protein

was included, labeled aggrecan in both the 4-h pulse and the 1-,

2-, and 3-day chase extracts formed very high molecular weight

complexes at both ages studied (Fig. 9, e and f).

These experiments highlighted the different results that can be

obtained using an alternative technique to study the same

process. For example, [35S]sulfate-labeled proteoglycan from

the 9-year-old’s specimen of cartilage (Fig. 5) was fully aggre-
Aggrecan Assembly in Normal Human Cartilage

gated in the pulse extract, whereas the 14-year-old's extract, analyzed by gel electrophoresis, had a mobility consistent with nonaggregated aggrecan. This was partly because of the methodology used, because when the 9-year-old's extract was electrophoresed in composite gels, it also appeared to contain 50% aggrecan (results not shown). The instability of aggregates in an electrophoretic field was also highlighted by the results shown by Fig. 9d: a faster migrating, diffuse molecular species was present in the chase cultures, and this could not be identified using gel chromatography. Thus, both methods (size exclusion chromatography and gel electrophoresis) are effective in describing the changes that take place under the different experimental conditions applied, but interpretation of the data depends on having a detailed knowledge of the molecules under investigation.

The endogenous aggrecan present in these extracts was also investigated by staining the gels with toluidine blue before subjecting them to autoradiography (results not shown). The electrophoretic pattern of these molecules showed that even in the pulse extracts, most of them were high molecular weight and therefore did not enter the composite gel and that this pattern did not change during the subsequent chase culture. However, treatment of the extracts of cartilage with either oligosaccharides of hyaluronan or link protein gave very similar patterns of toluidine blue staining to that observed for the newly synthesized aggrecan. It should be noted that both the endogenous and the newly synthesized aggregates from the mature cartilage were more unstable in the electrophoretic field than those extracted from immature cartilage.

Comparison of the Rate of Aggregation in Normal and Osteoarthritic Cartilage—Experiments were carried out to compare the extent of aggregation of aggrecan synthesized by cartilage explants taken from a normal knee joint (84 years) and from two osteoarthritic specimens (70 and 76 years). After pulse-labeling the cartilage explants with [35S]sulfate for 4 h, they were extracted directly with 4 M GnHCl, and after dialysis to promote the re-formation of aggregates, the extracts were eluted from a column of Sepharose CL2B. Considerably more of the radiolabeled aggrecan extracted from the osteoarthritic specimens was eluted in the void volume of the column (Fig. 10). In a subsequent study, the extent of aggregation of aggrecan in a 4-h pulse and in 2- and 4-h chase extracts were compared for a normal specimen of femoral condyle cartilage (75 years) and for an osteoarthritic specimen (73 years). Fig. 10 shows that acquisition of full aggregating potential was attained much faster by the newly synthesized aggrecan from the diseased specimen. The altered rates of assembly of aggrecan in the diseased cartilage was not a consequence of changes in the availability/extraction of hyaluronan, because analysis of the endogenous proteoglycans showed that 65–70% of the uronic acid was excluded from the column in each case.

DISCUSSION

Very little is known about the rate or the extent of assembly of aggrecan in situ. A number of questions remain unanswered about this process, and we have addressed these by providing some information about the extracellular organization of aggrecan. It is generally accepted that the stoichiometric relationship for the binding of aggrecan through its G1 domain to hyaluronan and link protein in an aggregate complex, is 1:1:1 (mol) (20). This is undoubtedly true for immature cartilage, which has been used to obtain much of the published experimental data. However, is the same relationship true for adult articular cartilage? The answer to this question has many repercussions for cartilage biology, particularly when one is considering the repair of cartilage defects by mature chondrocytes. Is all of the newly synthesized aggrecan processed in the same way? For example, are there different pools of aggrecan and are they present in different compartments of the tissue, in situ? It is our contention that age has a significant effect on all of these parameters.

The preliminary experiments carried out to investigate aggrecan structure were performed, not only to establish the techniques that would be used subsequently but also to provide the evidence to support a strategy that we have applied to improve our understanding of aging of normal articular cartilage, i.e. the need to advance our knowledge of both the endogenous and the newly synthesized molecules of cartilage. A subsequent report will deal with the composition and structure of endogenous aggrecan molecules in the various extracts of human cartilage.

The origin of the three toluidine blue-staining bands observed in the mature cartilage extracts will be discussed in detail in that publication, as will the immunoreactivity of them and their relationship to the radiolabeled molecules. However, it is pertinent to the discussion that follows here that our research makes clear that the two faster migrating bands could arise from the slower one by proteolytic modification during the relatively long time that these molecules are present in the extracellular matrix (2). It is also important to realize that they could be derived by a biosynthetic route. The recent study of chondroitin sulfation during aging of human articular cartilage (3) has gone some way to explaining the relationship between the three molecular species of aggrecan.

Although the differential extraction procedure described in this manuscript can give some very useful information about aggregation in situ on its own, it is still necessary to carry out an analysis of the structural properties of the molecules involved in order to make a statement about the organization of the complex. For example, although our findings are consistent with the extraction of a low affinity form of aggrecan from adult cartilage with PBS and its slow conversion to a high affinity...
form in situ, evidence is also presented that suggests that the nondissociative extract contains high affinity molecules that require some other stimulus for them to be able to interact with hyaluronan. A high proportion of newly synthesized aggrecan is extracted from immature cartilage with PBS, and yet much of it can form aggregates with low concentrations of hyaluronan. A far more likely explanation is that there is processing of macromolecules, such as hyaluronan, from a PBS-soluble form to one requiring guanidine for extraction, or that other matrix molecules are required to induce aggregation. This hypothesis is consistent with autoradiographic studies that were performed on pulse-labeled and pulse-chase explants. One would not expect the newly synthesized molecules in the 4-h pulse explants of immature cartilage to be fixed in the pericellular compartment. How then could they move to the intercellular space during the chase culture? Once again, it is more likely that they have “matured” but have not found the appropriate biological signal/environment (positional and structural) to promote aggregation. It is also likely that there are other interactions occurring in situ, possibly with molecules that we would not normally associate with the formation of aggregates, of which we know very little. A good example of this is the age-related increase in the formation of nonenzymatic cross-linking of proteins that has been demonstrated in human articular cartilage (21, 22). The study of Sandy and Plaaas (12) gives another possible explanation of our findings. They showed that it was necessary to include detergent in the column buffers in order to inhibit self-association of aggrecan. Although similar experiments were not carried out in the present study of human aggrecan, they should be included in any subsequent analysis of this phenomenon.

Osteoarthritis is a joint disease characterized by an eventual loss of the articular cartilage. During the progress of the disease, a decreased concentration of aggrecan in the tissue is often observed. Gray et al. (24) suggested that this altered composition would result in an increase in the pH of the extracellular matrix. It would become less acidic and, therefore, promote the conversion of aggrecan into a high affinity form (14). Obviously, this mechanism of control is countered by many other biological mechanisms, but it is pertinent that the rates of aggregation of aggrecan in osteoarthritic cartilage presented here are much faster than that measured for normal cartilage. Another feature of osteoarthritic cartilage is the variable status of the metabolism of the chondrocyte that is often observed. Some authors have stated that the rate of \(^{35}\)Ssulfate incorporation by these late stage specimens is invariably higher than normal (25), whereas others have shown that either there is no change from normal or proteoglycan synthesis is decreased (23). Whatever the finding is, the results serve to illustrate the phasic nature of the disease, even at this relatively late stage. It should be noted that all of the osteoarthritic specimens used in the present study had rates of \(^{35}\)Ssulfate incorporation that were within the normal range. It is also worth noting that unpublished studies\(^2\) have indicated that a decrease in the pO\(_2\) of the culture medium (to a value found in vivo decreases the rate of synthesis and delays the rate of aggregation. Thus, it appears that different biological mechanisms are active at different ages and that these directly influence the homeostasis of articular cartilage.

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\(^2\) S. Howat and M. T. Bayliss, unpublished data.
The Organization of Aggrecan in Human Articular Cartilage: EVIDENCE FOR AGE-RELATED CHANGES IN THE RATE OF AGGREGATION OF NEWLY SYNTHESIZED MOLECULES

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