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# Acquisition of hyaluronate-binding affinity *in vivo* by newly synthesized cartilage proteoglycans

John D. SANDY,* James R. O'NEILL and Linda C. RATZLAFF
Department of Orthopedics, Brown University, Rhode Island Hospital, Providence, RI 02902, U.S.A.

We have studied the hyaluronate-binding properties of aggregating cartilage proteoglycans synthesized *in vivo* by immature (6-week), mature (25-week) and aged (75-week) rabbits. Precursor isotope ($^{35}SO_4$) was given by intra-articular injection and articular cartilage was removed from rabbits after periods ranging from 1.5 h to 168 h. Proteoglycans were extracted with 4 M-guanidinium/HCl and monomers were isolated by CsCl gradient centrifugation under dissociative conditions. The percentages of both radiolabelled and total tissue monomers with a high affinity for hyaluronate [that is, capable of forming aggregates on Sepharose CL-2B in the presence of 0.8 % (w/w) hyaluronate] were then determined. For all samples about 30 % of the tissue monomers were high-affinity; however, less than 5 % of the radiolabelled monomers were high-affinity at 1.5 h after injection, and this figure increased gradually with time *in vivo*. The increase was rapid in immature rabbits, such that after 24 h, about 30 % of the radiolabelled monomers were high-affinity; on the other hand for mature and aged rabbits the increase was markedly slower such that 30 % high-affinity was attained only after about 72 h. The results show that aggregating cartilage proteoglycans are secreted *in vivo* in a 'precursor' form with a low affinity for hyaluronate, and suggest that conversion of these monomers to a form with a higher binding affinity occurs with a half-time of about 12 h in immature cartilages but greater than 24 h in mature cartilages. The possible relationship of these findings to the process of proteoglycan aggregation *in vivo* is discussed.

## INTRODUCTION

The aggregating proteoglycans of articular cartilage would appear to exist in the tissue in the form of multimolecular aggregates composed of proteoglycan monomers, link protein and hyaluronic acid (for review see ref. [1]). Assembly of these aggregates occurs following secretion of these components from chondrocytes, although the timing and sequence of the process remains to be firmly established [2].

It is now clear from biosynthetic studies with both cartilage explants [3–6] and chondrocyte cultures [7,8] that a high proportion of monomers can be secreted in a 'precursor' form with a 'low' binding affinity for hyaluronate, and that this form can be converted in the extracellular space to one of high binding affinity. Since the hyaluronate-binding properties of the proteoglycan core protein reside in an *N*-terminal globular domain which is dependent on disulphide bonding for its function [1], it seems likely that these different affinity forms are due to variability in the extent or arrangement of disulphides, although the evidence for this remains indirect [9].

Experiments in which the oxygen tension of the culture system [6] or the pH of the medium [7] were varied have shown that this binding affinity may be markedly altered by the conditions of culture and the method of preparation of samples for analysis. It therefore seemed possible that the appearance of low-affinity monomers in these systems might be a chondrocyte response to *ex vivo* conditions, rather than the result of normal chondrocyte biosynthetic activity. To investigate this we have now examined the hyaluronate-binding properties of newly synthesized monomers and turnover products isolated from the articular cartilage of rabbits radiolabelled *in vivo*. In addition, we have studied the effect of maturation and aging on these properties.

## EXPERIMENTAL

### Materials

Materials were obtained as previously described [7,10]. In addition, hyaluronic acid (Healon) was obtained from Pharmacia and bovine link protein was a gift from Dr. L. Rosenberg, Montefiore Hospital, New York, U.S.A.

### Radiolabelling *in vivo* and isolation of proteoglycan monomers

A total of 25 New Zealand White rabbits (at 6, 25 or 75 weeks old) were studied in five separate groups (see Table 1). Rabbits were anaesthetized with ketamine and given bilateral injections in hind knee joints of $^{35}SO_4^{2-}$ (0.25 ml of 1.0 mCi/ml in phosphate-buffered saline). Animals were killed at intervals ranging from 1.5 h to 168 h after injection and the articular cartilage from tibial and femoral surfaces of both hind knees was dissected into cold phosphate-buffered saline, sliced, washed and immediately extracted for 48 h at 4 °C in 4 M-guanidinium chloride / 50 mM-Na acetate / 10 mM-MES, 5 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride/5 μg of pepstatin/ml, pH 6.5. Typically, 650–850 mg (wet weight) of cartilage was obtained from each rabbit and this was extracted in 15 ml of extractant per

* To whom correspondence should be sent, at current address: Shriners Hospital, Tampa Unit, 12502 North Pine Drive, Tampa, FL 33612, U.S.A.

g of tissue. The extract was recovered by filtration on glass wool and the tissue was digested with papain. Portions of the extract and tissue digest were fractionated on PD-10 columns to determine total incorporation of isotope into macromolecules and the percentage extraction of proteolglycans with guanidinium chloride. Extracts were adjusted to a density of 1.45 g/ml with solid CsCl, centrifuged at 38000 rev./min ($r_{av.}$ = 8.02 cm) for 72 h and 12 °C and the D1 monomers isolated at a density greater than 1.54 g/ml. D1 samples (2.5 ml containing about 3 mg of proteoglycan) were dialysed against 0.15 M-Na acetate, pH 6.8, for 24 h at 4 °C before analysis and chromatography.

### Aggregation studies

Portions of D1 samples (containing 250 $\mu$g of total proteoglycan and about 25000 c.p.m. of radioactivity) were fractionated on Sepharose CL-2B columns (0.6 cm × 100 cm) eluted at 1.5 ml/h in 0.5 M-sodium acetate, pH 6.8, containing 0.1% (v/v) Triton X-100, collecting 0.5 ml fractions. To determine the percentage of high-affinity monomers (see below for definition), samples were run after addition of 0.8% (w/w) Healon and aggregation for 2 h at 22 °C. To determine maximum aggregability, samples were adjusted to 4 M-guanidinium chloride; hyaluronate (5% w/w) and link protein (5% w/w) were added and the whole was dialysed for 16 h at 4 °C against 0.15 M-Na acetate, pH 6.8, before chromatography. To determine maximum aggregability following alkaline pretreatment, samples were dialysed against 0.1 M-Tris/0.05 M-Na acetate, pH 8.6, for 48 h and 22 °C before aggregation with excess hyaluronate and link protein as above. To simplify graphical presentation, the radioactivity (c.p.m.) and colorimetric data ($A_{525}$) from each column analysis (see Figs. 1–5) were divided by the maximum values obtained for that analysis and presented as a percentage of the maximum. Percentages of aggregate were determined by cutting and weighing of profiles, assuming aggregate to elute in the void volume of the column as depicted by the hatched area shown on Figs. 1–5. To determine weight ratios of reactants the concentration of hyaluronate in Healon was taken as given by Pharmacia (10 mg/ml); the concentration of proteoglycan was not determined in absolute terms but as chondroitin sulphate equivalents, by reaction with dimethyl Methylene Blue as described [11] and with chondroitin 6-sulphate from shark cartilage as a standard; the concentration of link protein was determined by absorbance at 277 nm [12].

### Definition of high-affinity and low-affinity proteoglycans

Throughout this paper we define those monomers (total tissue or radiolabelled) which form aggregates with 0.8% (w/w) Healon on Sepharose CL-2B as high-affinity and those which fail to form aggregates as low-affinity. Under these conditions, the amount of hyaluronate provided is apparently insufficient to accommodate all monomers so that only molecules of high affinity are recovered in aggregates [6]. The terms 'high' and 'low' are used here to simplify description of the results only; the presence or absence of two discrete affinity populations in these samples remains to be established.

## RESULTS

### Radiolabelling *in vivo* and isolation of proteoglycans

Proteoglycans were isolated from the articular cartilages of rabbits labelled *in vivo* by extraction for 48 h in a buffer containing 4 M-guanidinium chloride and proteinase inhibitors. For all animals, the percentage extraction of total tissue proteoglycans was between 62% and 76%, with the range being significantly lower for the 75-week-old rabbits (62–68%) than for the younger animals (67–76%). The extractability of newly synthesized molecules was similar for all age groups, ranging between 77% and 83%, and this figure was not markedly influenced by the period between intra-articular injection and isolation of proteoglycans.

Proteoglycans were purified from these extracts by dissociative gradient centrifugation at a starting density

**Table 1. The specific radioactivity of monomers isolated from rabbits labelled *in vivo***

Groups of rabbits aged 6 weeks (Groups 1 and 2), 25 weeks (Groups 3 and 4) and 75 weeks (Group 5) were given intra-articular injections of $^{35}SO_4^{2-}$ and killed after periods ranging from 1.5 h to 168 h. Rabbits within each group were injected with a single isotope preparation, and cartilage extracts were processed and analysed as a group. Proteoglycan monomers were isolated by CsCl gradient centrifugation and their specific radioactivities (c.p.m./$\mu$g of proteoglycan) determined. The figures shown were calculated from triplicate analysis of D1 samples from single animals. Details of radiolabelling *in vivo* and proteoglycan isolation are given in the Experimental section.

| | | Specific radioactivity (cpm/$\mu$g of proteoglycan) | | | | |
|---|---|---|---|---|---|---|
| | Age... | 6 weeks | | 25 weeks | | 75 weeks |
| Period after injection (h) | Group... | 1 | 2 | 3 | 4 | 5 |
| 1.5 | | 154 | 98 | 154 | 106 | 143 |
| 3 | | – | – | 141 | – | – |
| 5 | | 173 | – | – | – | – |
| 6 | | – | – | 120 | – | – |
| 8 | | 164 | – | – | – | 115 |
| 24 | | 161 | 113 | 115 | 70 | 65 |
| 48 | | – | 81 | – | – | 55 |
| 72 | | – | 93 | 112 | 86 | 63 |
| 168 | | – | 48 | 114 | 82 | 41 |

of 1.45 g/ml. This low initial density was necessary to obtain a high recovery of both total tissue and radiolabelled monomer from all ages of rabbit; thus greater than 82% recovery was achieved in the D1 fraction (at 1.54 g/ml) in all samples described here. The specific radioactivity (c.p.m./$\mu$g of glycosaminoglycan) of the isolated D1 proteoglycans was determined at each labelling period in the five groups of animals studied (Table 1). The near-maximal specific activity obtained at 1.5 h in all cases shows that essentially all of the proteoglycan labelling occurred as a 'pulse' very soon after intra-articular injection, and that there was no further incorporation with time; this would be expected here since $^{35}SO_4^{2-}$ should be rapidly lost from the joint space by free diffusion into the circulation. Rapid disappearance of precursor isotope from the joint space was indeed indicated by the finding that all of the guanidinium chloride extracts, even at 1.5 h after injection, were essentially devoid of free isotope, as shown on PD-10 analysis.

At time periods up to 24 h, the specific activity obtained for individual animals in any one group (see groups 1 and 3 in particular) was very similar, demonstrating a high degree of reproducibility for the injection protocol and proteoglycan isolation procedures. It was of interest that the specific activity at 1.5 h was similar at all ages, which might indicate that the synthetic rates *in vivo* were also similar; however the labelling rate *in vivo* would be markedly affected by isotope supply to the cells which would itself be influenced by age-related changes in joint size, synovial fluid volume, etc., so that the specific activity values may not be an accurate measure of chondrocyte biosynthetic activity. All groups showed a marked decrease (up to 50%) in the specific activity of isolated monomers at 168 h relative to the 1.5 h value, consistent with the presence, in the articular cartilage at all ages, of a major pool of monomer with a rather short half-life.

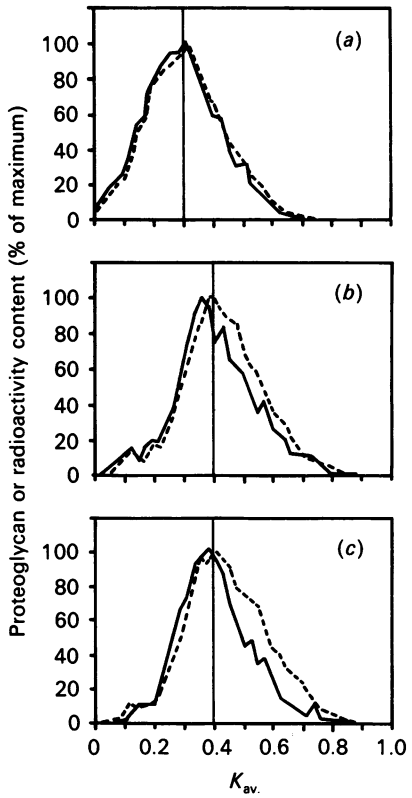


**Fig. 1. Effect of age on the size distribution of proteoglycan monomers from rabbit articular cartilage**

Portions of monomer samples from rabbits of different ages labelled *in vivo* were fractionated on Sepharose CL-2B (see Experimental section for details) and fractions were assayed for radioactivity (——) and proteoglycan (– – –). (*a*) 6 weeks; (*b*) 25 weeks; (*c*) 75 weeks.

### Age-related changes in monomer size

To establish the purity of D1 samples, preparations from 6-week, 25-week and 75-week-old animals were fractionated on Sepharose CL-2B columns without addition (Fig. 1*a*, *b* and *c* respectively); the profiles, which describe both the tissue and the radiolabelled proteoglycans in 1.5 h samples, have been plotted against $K_{av.}$ to illustrate age-related changes in monomer size. At all ages the proteoglycans were eluted almost entirely in the monomer position with little or no evidence for the presence of aggregates; this indicates that the CsCl gradient isolation removed all endogenous hyaluronate from these samples. At 6 weeks (Fig. 1*a*), the tissue and radiolabelled monomers were essentially identical, being eluted with a peak $K_{av.}$ of about 0.3; on the other hand at 25 weeks and 75 weeks (Figs. 1*b* and 1*c*) the size of tissue monomers (peak $K_{av.}$ of about 0.4) was smaller than that of the equivalent radiolabelled molecules, which were eluted with a peak $K_{av.}$ of about 0.37. Thus the mean hydrodynamic size of tissue monomers decreased markedly from 6 to 25 weeks with no further marked change up to 75 weeks. Further, the size of newly synthesized monomers clearly decreased from 6 to 25 weeks, but this change was slightly less pronounced than the change in tissue monomer size.

The results therefore confirmed the marked decrease in hydrodynamic size previously seen with aging from 6 weeks to 75 weeks in the rabbit [10]. The finding that the size of newly synthesized molecules was also markedly reduced as early as 25 weeks indicates that the decrease in tissue monomer size may be due to a turnover process involving the synthesis and deposition of small monomers by mature cells, rather than the accumulation in the tissue of partially degraded molecules.

### Hyaluronate-binding properties of monomers

The hyaluronate-binding affinities (see Experimental section for definition) of tissue and radiolabelled monomers in all samples were compared by chromatography on Sepharose CL-2B following aggregation in the presence of 0.8% (w/w) hyaluronate. The results for 6-, 25- and 75-week-old rabbits are shown in Figs. 2,3 and 4 respectively, and each panel shows the percentage of radiolabelled monomers recovered in aggregates (that is, in the high-affinity form).

In these samples, between 25 and 30% of tissue monomers were recovered in the high-affinity form, and this was the case at all ages and at all time points. In contrast to the tissue monomers, the percentage of radiolabelled monomer in the high-affinity form was near to zero at 1.5 h for all ages, and this figure increased gradually with time after intra-articular injection. Thus in 6-week-old rabbits (Fig. 2) the amount of high-affinity monomer increased to a maximum of 32% by 24 h; on the other hand in 25- and 75-week-old rabbits (Figs. 3

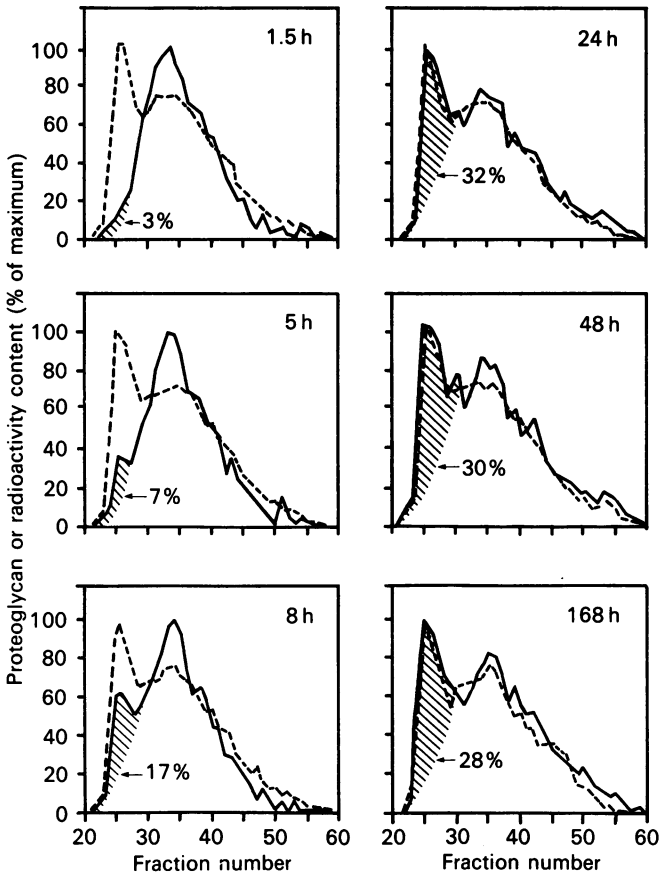


**Fig. 2. Effect of time after injection of precursor isotope on the hyaluronate-binding properties of monomers from immature rabbits**

Portions of monomer samples from 6-week-old rabbits (see Table 1) were fractionated on Sepharose CL-2B in the presence of 0.8% (w/w) hyaluronate, and fractions were assayed for radioactivity (——) and proteoglycan (---). The profiles shown were from rabbits in Group 1 (1.5,5,8 and 24 h) and Group 2 (48 h and 168 h). The profiles obtained for the 1.5 h and 24 h rabbits in Group 2 (not shown) were essentially identical to the 1.5 h and 24 h profiles shown. Each panel shows the period (h) between intra-articular injection and the death of the animals, and the percentage of the radiolabelled monomers present in aggregates (hatched area). Derivation of ordinate units as % of maximum is described in the Experimental section.

and 4) a similar increase to about 30% high affinity occurred, but this was not reached until much later, at about 72 h after injection. At no age was there a marked change in percentage of high-affinity monomers between 72 h and 168 h after injection.

### Aggregability of monomers with excess hyaluronate and link protein

The capacity of monomers in these samples to form aggregates on Sepharose CL-2B in the presence of excess hyaluronate and link protein was determined next and is described in Table 2. Under these conditions, all monomers with the capacity to bind hyaluronate, whether of low or high affinity , should be recovered in aggregate form. At all ages, and at all time points between 62% and 72% of tissue monomers were recovered in aggregates, showing that the aggregability of tissue monomers is not markedly affected by maturation or aging in the rabbit. In 6 week-old rabbits the aggregability of radiolabelled monomers was also high at all time points and similar to that of tissue monomers.

In 25- and 75-week-old rabbits, the aggregability of radiolabelled monomers was similar to the equivalent tissue molecules at 24 h and 72 h after injection, but considerably reduced at very early time periods (up to 8 h). This difference at early time periods is shown in Fig. 5 where the profiles of the 1.5 h samples from 6-week-old (*a*), 25-week-old (*b*) and 75-week-old (*c*) rabbits are shown. Moreover, the binding properties of the non-aggregating species shown in Figs. 5(*b*) and 5(*c*) were essentially unchanged by pretreatment of samples at pH 8.6 before addition of excess hyaluronate and link

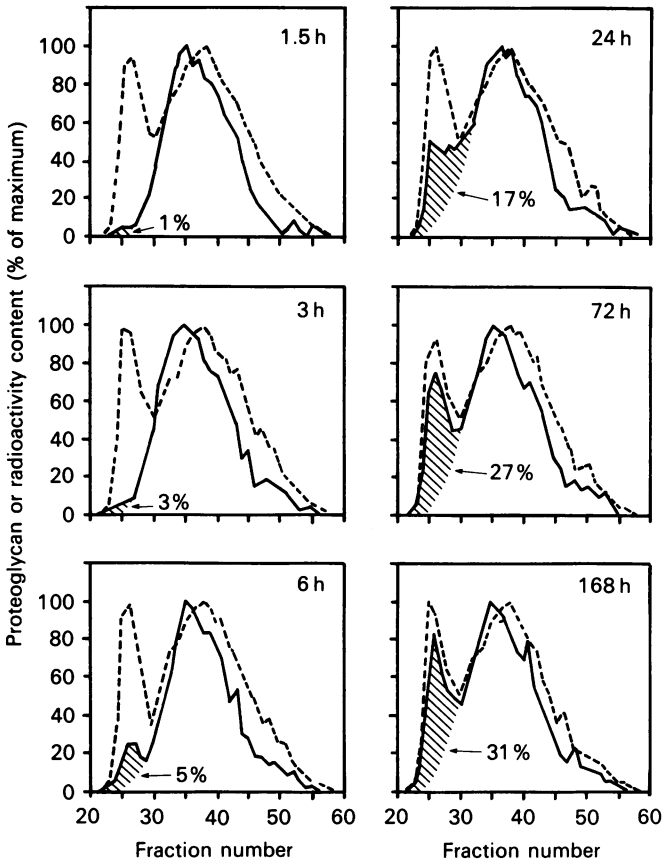


**Fig. 3. Effect of time after injection of precursor isotope on the hyaluronate-binding properties of monomers from mature rabbits**

Portions of monomer samples from 25-week-old rabbits (see Table 1) were fractionated on Sepharose CL-2B in the presence of 0.8% (w/w) hyaluronate, and fractions assayed for radioactivity (——) and proteoglycan (---). The profiles shown were all from rabbits in Group 3. The profiles obtained for Group 4 rabbits at equivalent times (not shown) were essentially identical to those shown. Each panel shows the period (h) between intra-articular injection and the death of the animals, and the percentage of the radiolabelled monomers present in aggregates (hatched area). Derivation of ordinate units as % of maximum is described in the Experimental section.

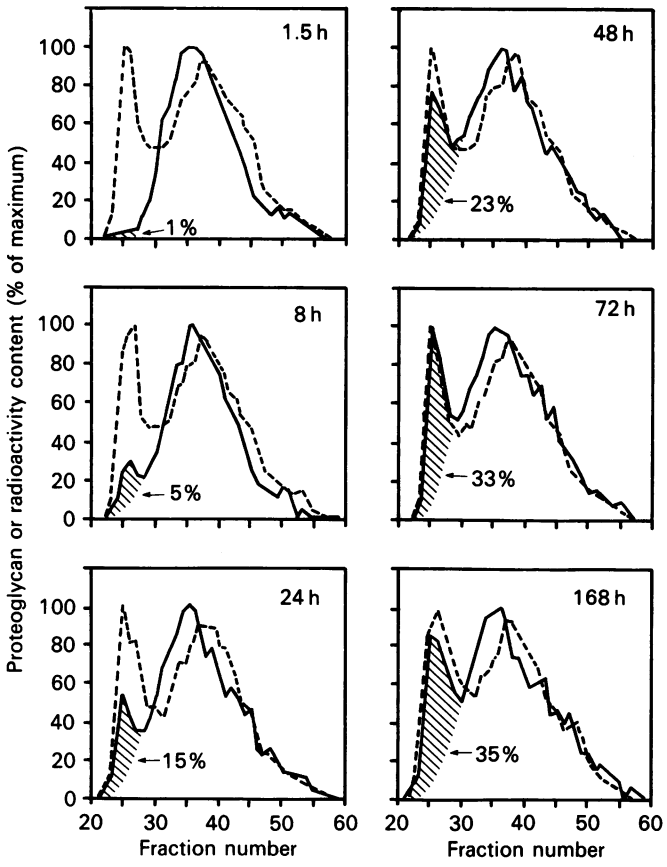


**Fig. 4. Effect of time after injection of precursor isotope on the hyaluronate-binding properties of monomers from aged rabbits**

Portions of monomer samples from 75-week-old rabbits (Table 1, Group 5) were fractionated on Sepharose CL-2B in the presence of 0.8% (w/w) hyaluronate, and fractions assayed for radioactivity (——) and proteoglycan (---). Each panel shows the period (h) between intra-articular injection and the death of the animals, and the percentage of the radiolabelled monomers present in aggregates (hatched area). Derivation of ordinate units as % of maximum is described in the Experimental section.

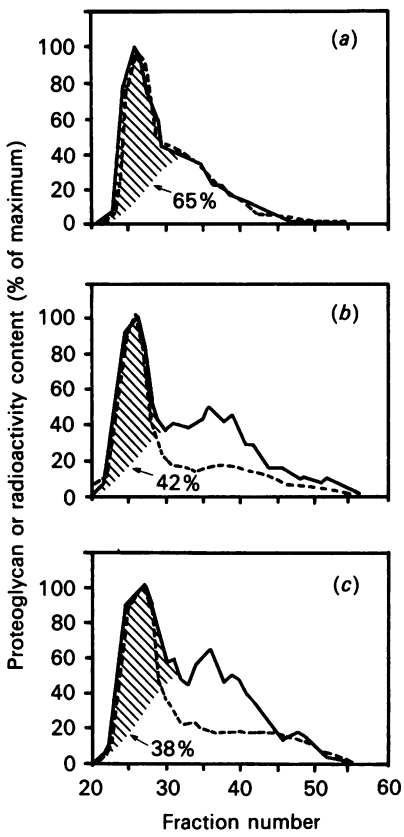


**Fig. 5. Aggregability of monomers with excess hyaluronate and link protein**

Portions of monomer samples, isolated from rabbits killed 1.5 h after injection (see Table 1), were added to excess hyaluronate and link protein under dissociative conditions, brought to associative conditions and fractionated on Sepharose CL-2B (see the Experimental section for details). Fractions were analysed for radioactivity (——) and proteoglycan (---), and each panel shows the percentage of radiolabelled monomers present in aggregates (hatched area). The profiles shown are from rabbits in (*a*) Group 1, (*b*) Group 3 and (*c*) Group 5. Derivation of ordinate units as % of maximum is described in the Experimental section.

**Table 2. Determination of the maximum aggregability of monomer preparations**

Proteoglycan monomer preparations derived from rabbits described in Table 1 were tested for maximum aggregability in the presence of excess hyaluronate and link protein. The samples from 6-week-old rabbits were from Group 1 (1.5,8,24 h) and Group 2 (72 h); the samples from 25-week-old rabbits were from Group 3 and the samples from 75-week-old rabbits were from Group 5. The table shows the percentage of aggregates achieved with the total tissue monomers and the newly synthesized ($^{35}S$) monomers in each sample. The figures shown were calculated from duplicate analysis of D1 samples from single animals. Details of methods of aggregation and chromatography are given in the Experimental section.

| | | Percentage aggregability with excess hyaluronate and link protein | | | | | |
|---|---|---|---|---|---|---|---|
| | Age... | 6 weeks | | 25 weeks | | 75 weeks | |
| Period after injection (h) | Monomer... | $^{35}S$-labelled | Tissue | $^{35}S$-labelled | Tissue | $^{35}S$-labelled | Tissue |
| 1.5 | | 62 | 68 | 42 | 72 | 38 | 68 |
| 6 | | – | – | 51 | 68 | – | – |
| 8 | | 61 | 62 | – | – | 49 | 66 |
| 24 | | 58 | 65 | 65 | 65 | 55 | 65 |
| 72 | | 65 | 62 | 64 | 69 | 64 | 71 |

protein (see the Experimental section). Such alkaline treatment has previously been shown [7] to markedly improve the hyaluronate-binding properties of newly synthesized monomers from chondrocyte cultures. Taken together these results suggest that in mature and aged rabbits a proportion of newly synthesized monomers rapidly lose, or never acquire, the ability to bind to hyaluronate.

## DISCUSSION

The results described here clearly demonstrate that the secretion of proteoglycan monomers with a low affinity for hyaluronate is a normal biosynthetic activity of articular chondrocytes, and one which occurs *in vivo* in both immature and mature cartilages. The results (Figs. 2, 3 and 4) also indicate that this 'precursor' form is slowly converted in the extracellular matrix to one of a higher affinity, so that newly secreted molecules gradually assume binding properties which are characteristic of the tissue pool.

The conclusion that conversion occurs in immature cartilage *in vivo* follows simply from the specific radioactivity figures (Table 1) and binding studies (Fig. 2); thus there is no marked change in specific activity between 1.5 h and 24 h after injection and yet the binding affinity increases markedly, consistent with a change in the binding properties of a single pool of monomers. On the other hand, with mature or aged rabbits the interpretation of the results may be more complex; with these samples the specific activity of the monomer pool consistently decreased during the period between 1.5 h and 24 h (Table 1), and this opens up the possibility that the increase in affinity seen over this period might be due to loss from the tissue of a sub-population of poorly-aggregating species.

Evidence for such a population of newly secreted monomers in mature and aged rabbits was indeed obtained from studies on maximum aggregability (Fig. 5 and Table 2); thus samples prepared from rabbits up to 8 h after injection of isotope contained a proportion of monomers which did not form aggregates even in the presence of excess hyaluronate and link protein. Further, these molecules failed to form aggregates following exposure to pH 8.6 under conditions previously shown to convert low-affinity monomers to the high-affinity form [7]. Such species were not seen in equivalent preparations from immature rabbits, and were not present in mature preparations after 24 h of processing *in vivo*. These non-aggregating molecules might therefore represent a turnover pool with a very short half-life which undergoes limited proteolysis soon after synthesis and thereby loses the capacity to bind to hyaluronate. In this regard it is interesting that similar evidence for an age-related increase in the susceptibility of newly-synthesized monomers to proteolysis was seen in our earlier work with rabbit cartilage explants [10].

Loss of non-aggregating species from mature cartilages *in vivo* would not however seem to account fully for the affinity increase observed here. Thus, major loss of this population was seen in the first 24 h after injection (Table 2), whereas a marked increase in the percentage of high-affinity monomers occurred between 24 and 72 h (Figs. 3 and 4). It therefore seems likely that conversion of low-affinity to high-affinity monomers occurs in mature cartilages, but that the rate is considerably slower than in immature tissue.

Experiments with low-affinity monomers purified from articular chondrocyte cultures (J. D. Sandy & A. H. K. Plaas, unpublished work) have indicated that conversion from low to high affinity can occur slowly at physiological pH when monomers are incubated in aggregates with excess hyaluronate. If the increase in affinity seen in the present study occurs after aggregation *in vivo* then the results would suggest that the rate at which newly-secreted monomers enter aggregates decreases substantially during maturation. Indeed, such a maturation-related decrease in the efficiency of aggregate assembly was concluded from our previous studies on the synthesis of monomer and link protein by chondrocytes from immature and mature rabbits [13].

Finally, efficient and rapid aggregate assembly, possibly on newly synthesized hyaluronate, would seem essential in the immature animal, where the cartilage matrix is undergoing rapid expansion and remodelling; on the other hand in the mature animal, where chondrocytes function to maintain a matrix of constant volume and composition, low-affinity monomers may diffuse through the intercellular space before 'finding' an available hyaluronate-binding site and before being converted to the high-affinity form. The precise role which the extracellular conversion of monomer from low to high affinity plays in these processes however remains to be established.

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