THE SYNTHESIS OF LOW-MOLECULAR WEIGHT PROTEOGLYCANS IN RABBIT-EAR CARTILAGE

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1. Introduction

We reported earlier [1] that the proteo-chondroitin sulphate complex of rabbit ear carilage is heterogeneous and comprises discrete low-molecular weight species, as minor constituents. These accounted for approx. 1.2% of the total proteoglycan content of the tissue. Both the Stokes radii and the molecular weights of three of these complexes showed a 1:2:3 relationship. It was postulated that the smallest macromolecules ($\bar{M}_w$ 3.8 X 10^4), referred to as F3, consisted of two chondroitin sulphate chains linked to a short polypeptide. The other two species (F1 and F2), which exhibited M.W. 7.7 X 10^4, respectively, were interpreted as comprising of four and six glycosaminoglycan chains.

In the present study, the variation in concentration of these low-molecular weight proteoglycans and their rate of ^{35}S-sulphate uptake have been determined *in vivo* during active glycosaminoglycan synthesis. This was to ascertain whether they represented degradation products of large macromolecules of the cartilage matrix or biosynthetic intermediates.

2. Materials and methods

2.1. *Treatment of animals for experiments*

A group of 40 New Zealand albino rabbits, 6–8 weeks old, were injected in the marginal ear vein with 1 ml of a 2% aqueous solution of crude papain [2]. It has been demonstrated that maximum depletion of cartilage matrix occurs 12 hr after papain injection and that the cellular biosynthetic activity normally starts compensating for losses after a further 12 hr [3].

To avoid contamination of newly synthesized proteoglycan with enzymic degradation products still present in the tissue, papain-treated rabbits were injected intramuscularly with cortisone acetate, at a dose of 5 mg/kg body weight [2], immediately after the papain injection and thereafter at 24 hr intervals for 5 days. This cortisone treatment was used to suppress cartilage proteoglycan biosynthesis [2, 4–7] until complete elimination of degradation products had occurred, as verified by analysis of the glycosaminoglycan level in the serum [8]. Rabbits were sacrificed, in groups of ten, at respectively 36, 40, 48 and 72 hr after the last corisone injection.

Thirty rabbits were treated with papain and cortisone as described above. Each animal was injected intraperitoneally with 2 mg of $Na_2\ ^{35}SO_4$, 40 hr after the last cortisone administration. All animals were then sacrificed, in groups of ten, after 30 min, 1 hr and 5 hr.

2.2. *Isolation and purification of cartilage proteoglycan*

The proteoglycan was extracted from an acetone-dried powder of ear cartilage by low-speed homogenization in water at 4° for 1 hr [1]. The homogenate was centrifuged at 5000 *g* for 15 min and the supernantant treated with 2 vol of ethanol. After a further centrifugation, KAc was added and the material that precipitated at 4° was collected after 24 hr. This extract was dissolved in 0.5 M KCl and fractionated by high-speed centrifugation [9]. The proteoglycan was recovered by alcohol precipitation and stored under reduced pressure. The residues of the water extraction and of the high-speed centrifugation of each preparation were pooled and suspended in 2 M $MgCl_2$ containing

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Table 1
Yields of water- and cetylpyridinium-extracted proteoglycans from rabbit ear cartilage.

Sample	Time elapsed after cortisone injection (hr)	Water-extracted material *	Cetylpyridinium-extracted material *
Control		420	7290
A	36	579	5300
B	40	547	5390
C	48	520	6170
D	72	445	7040

* Values are expressed as μg of hexuronic acid/g of dry carilage.

1% cetylpyridinium chloride. After homogenization at 50° for 1 hr and centrifugation at 38 000 *g* for 1 hr at 40°, the supernatant was diluted to a concentration of 0.6 M $MgCl_2$ [10]. The precipitate that formed was collected and dissolved in 2 M $MgCl_2$. Dissociation from cetylpyridinium chloride was carried out by extraction with chloroform–pentan-1-ol (5:4, v/v) [10]. Proteoglycan was recovered from the solution by the addition of ethanol.

2.2. *Gel filtration*

Gel filtration was carried out on a column of agarose-4% (bed volume 124 ml) equilibrated and eluted with 1 M KAç, pH 7. Samples, dissolved in 1 M-KAc, were applied to the column. The effluent fractions (3 ml each) were monitored for glycosaminoglycan by determination of their hexuronic acid content [11].

2.3. *Liquid scintillation counting*

Fractions collected after gel filtration of the preparations isolated from radiosulphate-treated rabbits were emulsified in a scintillant containing Triton X-100 and counted at 4° [12].

2.4. *Infrared absorption*

Infrared absorption spectra of the samples were obtained on a Unicam 200G infrared recording spectrophotometer, using Nujol mulls.

3. Results

The release of chondroitin sulphate into the blood stream of the papain-treated rabbits was monitored over a period of 96 hr and normal levels were found to be restored after approx. 72 hr.

The yields of the preparations extracted from control and papain-treated cartilages are reported in table 1. When the water-extracted materials were analyzed by infrared spectroscopy, prominent bands were observed at 725, 852 and 930 cm^{-1}, indicating that the isomer was mainly chondroitin 4-sulphate [13, 14], although chondroitin 6-sulphate is also known to be present in the tissue [15, 16].

The elution profiles of the water-extracted hexuronic acid-containing material on agarose-4% are reported in fig. 1. The time noted in each section of the figure refers to the time elapsed between the last cortisone injection and the sacrifice of the animals. The area under each peak was integrated and the value obtained is reported above the corresponding fraction. The control in fig. 1 is identical to that already reported in a previous paper [1]. The cetylpyridinium-extracted proteoglycan behaved as an excluded fraction when chromatographed on agarose-4%.

The results of the radiosulphate incorporation into the water-extracted material are reported in fig. 2. The time indicated in each section now refers to the time elapsed between the injection of $^{35}SO_4^{2-}$ and the sacrifice of the animals. The proteoglycan extracted by cetylpyridinium chloride showed no significant radioactive-label incorporation in the 30 min and 1 hr experiments. After 5 hr, a specific activity of 3000 cpm/mg of hexuronic acid, similar to that of FO, was found.

4. Discussion

The proteoglycan solubilized by cetylpyridinium chloride was excluded during gel chromatography, indicating that the low-molecular weight fractions were completely extracted by homogenization in water. Therefore, the quantitative values reported in fig. 1 represent their true concentrations in the cartilage matrix.

Depletion of cartilage by papain enhances

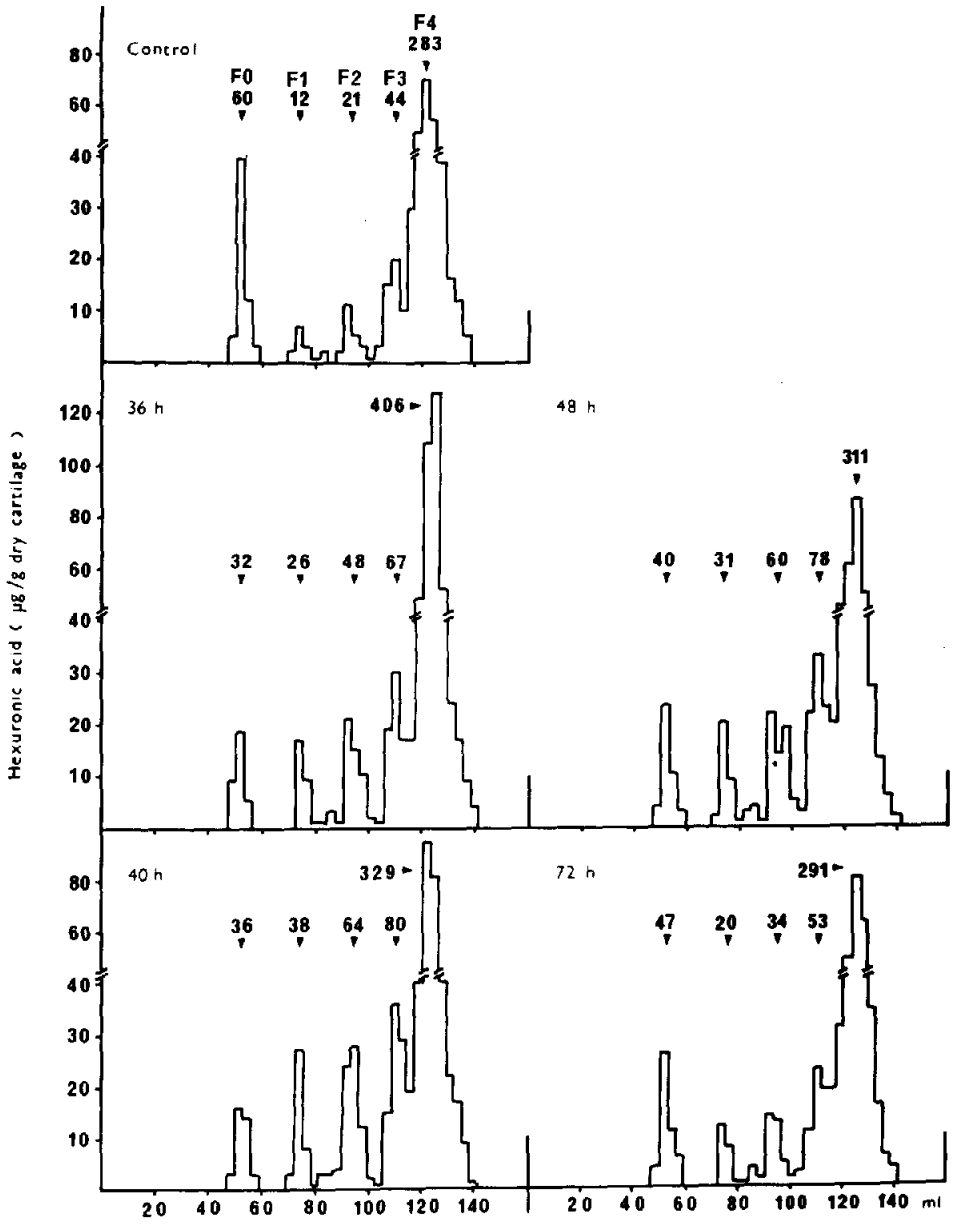

Fig. 1. Elution profiles of the water-soluble proteoglycan. Agarose-4%. Bed volume 124 ml, void volume 52 ml. Figures above represent areas of peaks (see text).

chondrocyte biosynthetic activity towards glycosaminoglycan production both *in vivo* and *in vitro* [17–21]. The elution profiles of the water-extracted material from papain-treated cartilage clearly show that all the gel-retarded fractions (F1, F2, F3, and F4) increased in concentration when compared with controls. It is of interest to note that this rise in concentration took place immediately the cortisone treatment was discontinued and reached a maximum after 40 hr. At 72 hr, values approaching those

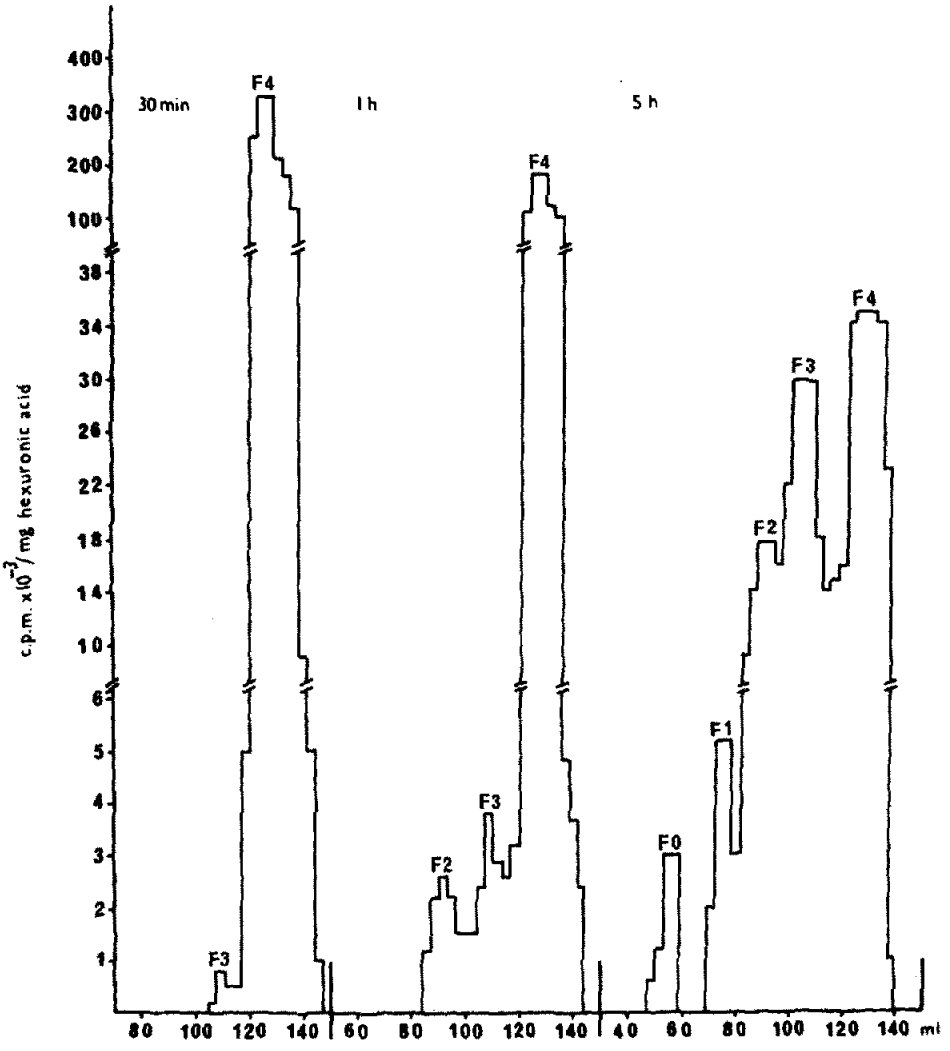

Fig. 2. Elution profiles of the water-soluble proteoglycan obtained from rabbits injected with $Na_2\ ^{35}SO_4$. Agarose-4%. Bed volume 124 ml, void volume 52 ml.

typical of controls were obtained. Meanwhile, fraction F0, the highly polymeric proteoglycan, slowly increased in concentration from a starting value of approx. 50% of the control level. The radiotracer experiments conducted on papain-treated rabbits 40 hr after the last cortisone injection, when the concentration of the low-molecular weight components was at a maximum in the tissue, show that label incorporation started from the low end of the molecular weight distribution profile. It should be emphasized that in the 5 hr experiment only, label was detected in F0 and in the material extracted by cetylpyridinium chloride which constitutes approx. 30% of the cartilage proteoglycan. These observations suggest that the low-molecular weight fractions represent macromolecular species normally present in the tissue and not breakdown products of larger protein-polysaccharide macromolecules.

From the information which is at present available, it is not possible to reach a firm conclusion on whether these macromolecules are themselves distinct terminal biosynthetic products or inter-

mediates in the synthesis of the highly polymeric cartilage proteoglycan(s). Due to possible large errors in the calculation of specific activities, caused by estimating low hexuronic acid concentrations, a precursor–product relationship between these species cannot be derived from data in fig. 2. However, the decrease of label from the low-molecular weight end of the gel chromatography profile and its appearance and rise in successive higher-molecular weight species is in keeping with the latter hypothesis.

It is thought that fraction F4, which is characterized by the highest rate of incorporation of ^{35}S-sulphate, may consist of newly synthesized and yet incomplete macromolecules. This view is supported by the observation that in rabbit ear cartilage chondroitin sulphate chains of M.W. less than 12 000, and therefore presumably incomplete, have been found to exhibit a specific activity higher than any of the chains of larger size [22].

Acknowledgements

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