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Replacement of Proteoglycans in Embryonic Chick Cartilage in Organ Culture after Treatment with Testicular Hyaluronidase

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Explants of cartilage from tibiae of 11–12 days chick embryos were grown in organ culture. To one group hyaluronidase was added to the medium during the first 2 days of culture; the treated tissue was then cultured in medium without enzyme for a further 4 days. Control explants grown in hyaluronidase-free medium for 6 days grew rapidly in size and the total hexosamine content more than doubled during this time. After exposure to hyaluronidase, much of the hexosamine was lost from treated cartilage and appeared in the culture medium, but it was mostly replaced in the tissue during the subsequent recovery period. Analysis of cartilage and medium showed that net synthesis of hexosamine increased greatly in treated cartilage. The proteoglycans were extracted by two procedures from control and treated cartilage after 2, 4 and 6 days in culture. The hydrodynamic sizes of the purified proteoglycans were compared by gel chromatography and the composition of the gel-chromatographic fractions was determined. The proteoglycans from controls did not change during culture, but after exposure to hyaluronidase the proteoglycans from treated cartilage were of much smaller size and lower chondroitin sulphate content. During recovery, even though new proteoglycans were formed, they were nevertheless of smaller size and lower chondroitin sulphate content than control proteoglycans. They gradually became more like control proteoglycans during recovery from treatment, but even after 4 days they were not yet the same. After 2 days of treatment with the enzyme, the chondroitin sulphate in the cartilage was of shorter chain length than in controls but during recovery after 4 and 6 days in culture, the chain lengths in control and treated cartilage were similar. It is concluded that the proteoglycans formed in embryo cartilage in response to their depletion by enzyme treatment contained fewer chondroitin sulphate chains attached to the protein moiety of proteoglycans. This may have resulted from a failure under stress to glycosylate the protein moiety to the usual extent; alternatively the synthesis of normal proteoglycans of low chondroitin sulphate content may have increased, thus changing the proteoglycan population.

The structural organization of cartilage results from an ordered pattern of several intercellular macromolecules such as collagen and proteoglycans; changes in this pattern, or in the nature of the macromolecules produced by the cells, could alter the character and properties of the tissue. Hence the elucidation of the mechanisms involved in such changes may well be of direct relevance to the understanding of factors involved in the development of certain pathological conditions.

Proteoglycans, which in cartilage are a particularly important intercellular constituent (reviewed by Muir, 1971, 1972), consist of glycosaminoglycans covalently linked to a protein backbone (Mathews & Lozaityte, 1958; Partridge *et al.*, 1961). These macromolecules are normally polydisperse in size and contain varying proportions of the major components: chondroitin sulphate, keratan sulphate and protein (Pal *et al.*, 1966; Rosenberg *et al.*, 1967; Tsiganos & Muir, 1969; Brandt & Muir, 1971).

The work of Fitton-Jackson (1970*a*) showed that treatment with testicular hyaluronidase of tibial rudiments grown in organ culture resulted in the loss of much of the glycosaminoglycans from the cartilage; during subsequent culture in the absence of hyaluronidase the concentration of constituents in controls was regained. The advantage of organ culture is that it is a closed system, and thus changes in the tissue and its environment can be assessed and the net synthesis of any product may be determined. The chondroitin sulphate moiety of the proteoglycans can be specifically degraded by hyaluronidase (Hoffman *et al.*, 1956) but hyaluronidase does not attack keratan sulphate; the degradative effect of the enzyme on cartilage proteoglycans is to produce a residual protein 'core' in which keratan sulphate

moieties remain intact, but chondroitin sulphate chains are lost, mainly leaving the specific linkage region containing neutral sugars through which the chains were originally attached to the protein core (Rodén, 1970). As chondroitin sulphate contains galactosamine and keratan sulphate contains glucosamine the preferential loss of chondroitin sulphate may be assessed from changes in the galactosamine/glucosamine molar ratios.

Since the biosynthesis of proteoglycans is intracellular (Hardingham & Muir, 1972), no repair of partially degraded extracellular proteoglycans is possible; hence any recovery from the effects of hyaluronidase treatment would imply the formation of new molecules. Whether the proteoglycans formed during recovery are the same as those present in untreated tissue in organ culture is examined here. Some preliminary results have been reported (Muir, 1971).

Experimental

Materials

All reagents were of analytical grade, with the exception of galactosamine hydrochloride, glucosamine hydrochloride, glucuronolactone, carbazole, guanidine hydrochloride (which was purified with activated charcoal; Norit NK, Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) and acetylacetone (which was redistilled: b.p. 133–134°C). Calf thymus DNA (sodium salt) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., supplied chondroitin sulphate (shark cartilage), testicular hyaluronidase (EC 3.2.1.35) and crude papain (EC 3.4.4.10). Twice-crystallized papain, 4 units/mg of protein, and hyaluronic acid (sodium salt) were obtained from BDH (Chemicals) Ltd., Poole, Dorset, U.K. Visking tubing (width 24/32in and 8/32in) for dialysis was obtained from Scientific Instruments Centre, London, W.C.1, U.K.

Methods

Analytical methods. Uronic acid was measured by an automated modification (D. Heinegård, personal communication) of the method of Bitter & Muir (1962) with glucuronolactone used as standard. Protein was measured by an automated modification (D. Heinegård, personal communication) of the method of Lowry *et al.* (1951) with bovine serum albumin fraction V as standard. In series A, tissue and medium were hydrolysed in 4M-HCl under the conditions described in detail by Fitton-Jackson (1970*a*), and subsequently hexosamine was determined by the procedure of Boas (1953) with columns of Dowex 50. In series B proteoglycans were hydrolysed in 8M-HCl under N_2 at 95°C for 3h (Swann & Balazs, 1966). Excess of acid was removed by rotary evaporation at 40°C and, after the addition of a known volume of water, hexosamine was measured by the method of Kraan & Muir (1957) with galactosamine hydrochloride as standard. Molar ratios of galactosamine/glucosamine were determined in the same hydrolysates dissolved in water, with a Locarte amino acid analyser. When total tissue hexosamine of series B was to be determined the dried tissue samples were hydrolysed as described above and the hexosamines partially purified before analysis by a procedure similar to that of Boas (1953).

The hydrolysate was decreased in volume by rotary evaporation at 40°C almost to dryness. Water (2ml) was added and the sample, together with 1ml of water to wash out the tube, was applied to a column (5.0cm×0.5cm) of Dowex 50 (H^+ form; X 8; 100–200 mesh). The column was washed twice with 2ml of 0.1M-HCl and the hexosamines were eluted with 6ml of 0.6M-HCl. Samples of the eluent were evaporated to dryness at 40°C with a rotary evaporator and then 0.5ml of water was added and the hexosamine content or the galactosamine/glucosamine molar ratio was determined as described above. The recovery of standard hexosamine samples from three such Dowex 50 columns was 98%, 101% and 99% respectively.

Determination of DNA. Fresh wet tissue samples were prepared essentially according to Schneider (1945) and subsequently analysed by means of the diphenylamine reaction of Burton (1956), as modified by Croft & Lubran (1965) and Giles & Myers (1965). Calf thymus DNA containing 8.36% DNA P was used as standard.

Measurement of hyaluronidase activity in the media. Samples of media, 1.0ml, were added to 2.0ml of 0.15M-sodium acetate, pH5.0, containing 0.98mg of hyaluronic acid/ml. The viscosity of the mixed solution (final pH5.3) was measured at various times (5–45min) after the addition of medium in an Ostwald capillary viscometer at 30°C. The initial rate of reduction in the viscosity of the solution was taken as a measure of the hyaluronidase activity. When the medium contained a high activity the determination was carried out with the medium diluted 1/20 with unincubated control medium containing no hyaluronidase. There was no decrease in viscosity over 60min when control medium alone was used in the assay.

Organ culture. The cartilaginous portions were dissected from the proximal and distal regions of tibiae from chick embryos at stage 37½ (Hamburger & Hamilton, 1951) and grown in organ culture on stainless-steel grids in the presence of 3ml of a chemically defined culture medium under an atmosphere of $O_2+CO_2+N_2$ (7:1:12) (Fitton-Jackson, 1970*a*,*b*).

Experimental explants were treated with 500μg of bovine testicular hyaluronidase (300 turbidity-reducing units/mg)/ml of medium for 48h from the beginning of culture and were then washed twice for at least 1h in standard medium and transferred to fresh culture medium for a recovery period of 4 days. Controls were cultured in a similar way for up to 6 days, but with hyaluronidase omitted from the starting medium. All media were changed at 24h intervals during the last 4 days of culture. Tissue samples were taken directly from the embryos and from treated and control explants after 2, 4 and 6 days in culture, and were kept at −20°C for subsequent analysis. All media from each period and washing were pooled and stored at −20°C before analysis.

Isolation of glycosaminoglycans from whole cartilage. Samples of cartilage were defatted with acetone–ether (1:1, v/v) overnight and dried in a vacuum desiccator. They were then suspended in 0.1M-sodium acetate buffer, pH5.5, containing 50mM-EDTA (disodium salt) and 10mM-L-cysteine at 60°C, and 20mg of activated (Kimmel & Smith, 1954) crude papain was added per g wet wt. of cartilage. After 4h of digestion, the small amount of insoluble residue containing a negligible amount of hexosamine was removed by centrifugation and samples of the supernatant were used to determine the total amount of chondroitin sulphate and the size distribution of the chains.

Extraction of proteoglycans (procedure I). Cartilage from tibiae, about 1.0g wet wt., was frozen and pulverized in a hammer press while cooled with solid carbon dioxide. The powdered material was transferred to 10ml of 2M-$CaCl_2$, pH5.8, in a stoppered 50ml centrifuge tube and was agitated on a mechanical roller for 48h at 4°C. The extract was centrifuged and the supernatant removed. The cartilage residue was washed in 5ml of 2M-$CaCl_2$ and centrifuged again. The washings and supernatant were combined and dialysed against 10vol. of 50mM-sodium acetate, pH5.8, overnight. Solid CsCl was added to adjust the density to 1.50g/ml and 18ml of each extract was then centrifuged at 95000$g_{av.}$ for 48h at 20°C in an MSE/65 centrifuge with a 8×25ml titanium rotor. The centrifuge tubes were frozen in an acetone bath containing solid carbon dioxide and sawn in two. The fraction from the bottom of the tube containing about 6ml and the upper fraction containing about 12ml were dialysed against a large excess of water and then against 0.5M-sodium acetate, pH6.8. The content of uronic acid was measured in the upper fraction and the contents of uronic acid, protein and the molar ratios of galactosamine/glucosamine were measured in the bottom fraction.

Extraction of proteoglycans with solutions of increasionic strength (procedure II: see Scheme 1). Samples of pulverized cartilage were suspended in 10 times their wet wt. of 0.15M-sodium acetate, pH6.8, and agitated overnight at 4°C and then centrifuged. From the supernatant 0.5ml was removed and the uronic acid content determined.

The cartilage, suspended in the same solution, to which 5M-$CaCl_2$, pH5.8, was added to bring the concentration of $CaCl_2$ to 0.25M, was agitated for 24h at 4°C. The solution was then centrifuged and 0.5ml taken from the supernatant for the determination of uronic acid content. This whole procedure was repeated twice more, the $CaCl_2$ concentration being raised to 1.0M and then to 2.0M. After the final centrifugation the supernatant was removed from the cartilage residue, which was washed with 2M-$CaCl_2$. The yield at each stage and the final yield was based on the uronic acid content of the extracts, allowing for the samples removed for analysis. The supernatant and washings were combined and the proteoglycans purified as described above for the extraction with 2M-$CaCl_2$.

Glycosaminoglycans remaining in the cartilage residues. The cartilage residues from both extraction procedures were agitated in water to remove excess of $CaCl_2$ and then digested with 20mg of activated crude papain/g as described above for the isolation of glycosaminoglycans from whole cartilage.

The uronic acid content of the papain digest was determined, as well as the hexosamine content after hydrolysis and elution from columns of Dowex 50, as described for total tissue hexosamine. With the same hydrolysates, galactosamine/glucosamine molar ratios were also determined as described above.

Gel chromatography. (*a*) Sepharose 2B. Samples of proteoglycans extracted by procedure I or II, containing 1mg of uronic acid in 1.0ml of 0.5M-sodium acetate, pH6.8, were applied to a column (180cm× 1.1cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden) eluted with 0.5M-sodium acetate, pH6.8, driven by a peristaltic pump at the rate of 5ml/h at 4°C. Fractions of 40 drops (about 2.5ml) were collected and their uronic acid contents determined. Fractions containing proteoglycans of largest (E), medium (R_1) and the smallest (R_2) sizes were pooled and analysed for uronic acid and protein. Samples were concentrated by vacuum dialysis and the galactosamine/glucosamine molar ratios determined after hydrolysis as described above. For the proteoglycans extracted from control cartilage by procedure I, fractions R_1 and R_2 were pooled to give a single fraction R, as the amount of proteoglycans in fraction R_2 was very small.

(*b*) On Sephadex G-200. Samples of the papain digests of whole cartilage containing about 1mg of uronic acid were applied to a column (120cm× 1.1cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) eluted with 0.2M-sodium acetate, pH6.8, under a hydrostatic pressure of 50cm. Fractions of 35 drops (about 2.2ml) were collected and their uronic acid contents determined.

Results

The results were derived from two series of experiments in which the dissected cartilaginous portions of chick tibiae were cultured under similar conditions. Series A samples were used to determine wet and dry weights, hexosamine and DNA contents of the cartilage at various times before and after culture with testicular hyaluronidase, and to determine the hexosamine content and hyaluronidase activity of the corresponding media. Cartilage samples from series B were used for the preparation of proteoglycans to compare their size and composition throughout the culture period.

Analysis of cartilage and media

The dry weight, wet weight and total hexosamine of samples from both control and hyaluronidase-treated cartilage, series A, after 0, 2, 4 and 6 days in culture, are shown in Table 1. Although the hexosamine content/mg dry wt. of control cartilage did not vary greatly during culture, the size of the explants increased considerably, as indicated by the wet and dry weights and total hexosamine content of the cartilage moiety of each rudiment. It is thus apparent that, in the controls, the total hexosamine content per rudiment increased more than 2½ times during culture (Table 3), but the hexosamine in the medium was only a small proportion of the total and did not increase. The DNA content also increased by about 20% during 6 days of culture, showing that the number of cells had risen, although to a smaller extent than the increase in total hexosamine (Table 3). The proteoglycans formed during this time were the same as those present initially *in ovo*; the distribution of macromolecular size (Figs. 1 and 2) and composition (see Tables 5 and 6) did not alter during culture. Furthermore the galactosamine/glucosamine molar ratio remained fairly constant (Table 2) and showed that chondroitin sulphate accounted for about 94% of the total glycosaminoglycans.

The effect of hyaluronidase led to a considerable loss of hexosamine from the cartilage into the medium (Table 3). After 2 days' exposure to the enzyme 85% of the total hexosamine of the system was in the medium (series A); presumably this was derived largely from the breakdown of chondroitin sulphate chains to oligosaccharides, which then diffused into the medium. During 2 and 4 days of recovery the hexosamine content of the tissue increased rapidly, finally reaching 79% of control values. Over this period, the proportion of the hexosamine in the medium was greater than in controls but was much lower than during treatment. From the analysis of tissue and medium the net synthesis of hexosamine per rudiment was calculated (Table 3). During treatment it was twice as high as in controls and remained so during the first 2 days of recovery. During the last 2 days of culture net synthesis was still 25% above the control value. Throughout the period of culture the DNA content of treated cartilage was below that of controls, showing that the increased synthesis of hexosamine was performed by fewer cells (Table 3).

The results from series A and B were in agreement (Tables 1 and 2). From the molar ratio of galactosamine to glucosamine remaining in the tissue (series B) it appeared that the loss of hexosamine into the medium during treatment was primarily galactosamine, since in treated rudiments the galactosamine content was only 19.5% of controls whereas the glucosamine content was still 93.3% of the control (Table 2). During recovery from treatment the galactosamine/glucosamine molar ratio increased in treated cartilage, but was still below that of the controls at the end of culture (Table 2).

The chondroitin sulphate chains in the tissue immediately after exposure to hyaluronidase were shorter than in control cartilage, as shown by gel

Table 1. *Analysis of control and hyaluronidase-treated cartilage (series A)*

Cartilage from 170 rudiments was used (five groups per determination).

	Total time in culture (days)	Wet wt. of cartilage (mg/rudiment)	Dry wt. of cartilage (mg/rudiment)	Wet wt./dry wt. ratio of cartilage	Hexosamine content of cartilage (μg/mg dry wt.; mean values)
Ex ovo	0	9.88±2.1	0.97±0.11	10.2	48.5
Control cultures	2	12.78±2.9	1.10±0.09	11.6	51.6
	4	18.4 ±1.7	1.56±0.051	11.8	54.5
	6	32.8 ±3.1	2.62±0.092	12.5	50.5
Hyaluronidase-treated cultures (Period of recovery)	2	8.32±2.7	0.93±0.091	8.9	17.0
	4	11.39±2.4	1.22±0.043	9,3	43.3
	6	21.59±3.2	1.97±0.092	11.0	53.5

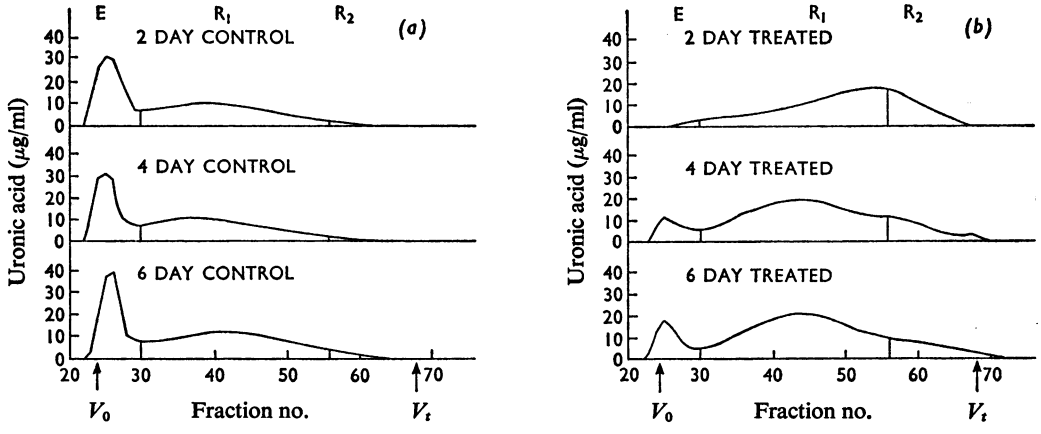


Fig. 1. *Elution from Sepharose 2B of proteoglycans extracted by procedure I from control (a) and hyaluronidase-treated (b) cartilage (series B)*

Proteoglycan samples from cartilage after a total of 2, 4 and 6 days in culture were applied to a column (180cm × 1.1cm) of Sepharose 2B eluted with 0.5M-sodium acetate, pH6.8, at the rate of 5ml/h at 4°C. The uronic acid content of each 2.5ml fraction was determined. As shown by the vertical lines fractions containing proteoglycans of largest (E), medium (R_1) and smallest (R_2) size were pooled and kept for analysis (Table 2). V_0 indicates the void volume and V_t the total bed volume of the column.

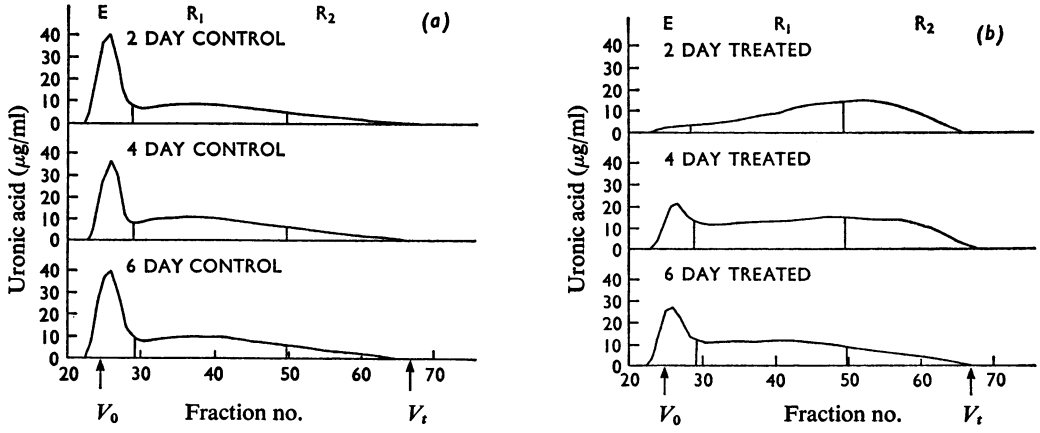


Fig. 2. *Elution from Sepharose 2B of proteoglycans extracted by procedure II from control (a) and hyaluronidase-treated (b) cartilage (series B)*

Proteoglycan samples from cartilage after 2, 4 and 6 days in culture were fractionated by gel chromatography exactly as described in Fig. 1. Fractions were pooled as shown by the vertical lines to give similar fractions E, R_1 and R_2, which were analysed (Table 4). V_0 and V_t are described in Fig. 1.

chromatography on Sephadex G-200 after removal of most of the protein with papain (Wasteson, 1969) (Fig. 3); most of the oligosaccharides that had been produced by the action of the enzyme, however, must have diffused out of the tissue, since papain digests of the whole tissue contained almost none (Fig. 3). During recovery, when the chondroitin sulphate was newly formed, 2 and 4 days after the cartilage was removed from the medium containing hyaluronidase, the chain lengths of the chondroitin sulphate were similar to those in control cartilage (Fig. 3). This suggested that there was no residual hyaluronidase left in the cultures during the period of recovery, and indeed estimation of the hyaluronidase

Table 2. *Analysis of control and hyaluronidase-treated cartilage (series B)*

	Total time in culture (days)	Hexosamine content of cartilage (μg/mg dry wt.)	Glucosamine content of cartilage (μg/rudiment)	Molar ratio $\frac{\text{galactosamine}}{\text{glucosamine}}$
Ex ovo	0	43.0	4.5	9.4
Control cultures	2	47.7	6.4	8.8
	4	46.9	7.5	10.3
	6	45.4	12.2	8.9
Hyaluronidase-treated cultures (Period of recovery)	2	18.2	6.0	1.84
	4	30.3	7.3	5.7
	6	39.5	10.1	7.6

in the medium showed that the activity was negligible (Table 4). Even during the first day of recovery it was only 0.16% of the original activity. The activity was measured by a sensitive viscometric method with hyaluronic acid as the substrate. It is to be noted that the enzyme is about 15 times more active towards hyaluronic acid than towards chondroitin sulphate (Meyer & Rapport, 1952), and that activity was estimated near the optimum pH, i.e. 5.3, whereas the cultures were maintained at pH7.4–7.8.

Comparison of proteoglycans (series B)

Extraction of control cartilage by either procedure I or II brought into solution about 85% of the uronic acid in the tissue (Tables 5 and 7), there being little difference in the size (Figs. 1 and 2) or composition (Tables 5 and 6) of the proteoglycans extracted by the two methods. On the other hand, with procedure I not more than about half the proteoglycans were extracted from treated cartilage immediately after exposure to hyaluronidase, although the proportion rose to 69% after 4 days of recovery (Table 5). With procedure II, however, where the extracting solution was initially of low ionic strength, 70–80% of the total uronic acid could be extracted from treated cartilage (Table 7). The proportion of the total uronic acid brought into solution, at each stage of procedure II, changed considerably during the period of recovery (Fig. 4), the proportion extracted at low ionic strength decreasing with time. It is possible that when treated tissue was extracted by procedure I with a solution of high ionic strength (2M-$CaCl_2$), the lower yield may have been the result of a 'salting-in' effect of 2M-$CaCl_2$ on proteoglycans that had lost much of their chondroitin sulphate from the action of hyaluronidase; alternatively, the lower yield may be due to some interaction or co-precipitation with other constituents of the cartilage matrix.

The proteoglycans extracted by both procedures from control and treated cartilage were compared after being purified by equilibrium density gradient centrifugation in caesium chloride (Tsiganos *et al.*, 1971). About 95% of the uronic acid was recovered from the bottom third of the gradient, in which there was almost no hydroxyproline. The elution profiles from Sepharose 2B showed that the proteoglycans had a broad distribution of molecular size (Figs. 1 and 2). There were some differences in the distribution of sizes and in the composition of the proteoglycans extracted by the two procedures, but these were small compared with the differences between the proteoglycans extracted from control and treated cartilage by either procedure. After 2 days of exposure to the hyaluronidase, the proteoglycans left in the tissue were smaller in size and contained more protein and glucosamine than controls (Figs. 1 and 2; Tables 5 and 6). During recovery the average size of the proteoglycans increased, but a distribution of molecular size similar to that of controls was never fully regained (Figs. 1 and 2) even though most of the proteoglycans would be newly formed, as there had been a fivefold increase in total hexosamine in the treated tissue (Table 2). Furthermore, although the galactosamine/glucosamine molar ratio increased during recovery, it was never restored to that of proteoglycans in control cartilage (Table 7). Even at the end of the period of recovery proteoglycans from treated cartilage contained not only more glucosamine but also more protein than those in control cartilage, the differences being most evident in the largest (E) and smallest (R_2) molecules (Tables 5 and 6).

Analysis of cartilage residue (series B)

Not all the hexosamine could be extracted by either procedure from control or treated cartilage. The galactosamine/glucosamine molar ratios of the residues were much lower than in the extracted proteoglycans (Table 7). There was little difference, however,

Table 3. *Analysis of control and hyaluronidase-treated cartilage and media and calculated net synthesis of hexosamine (series A)*

Cartilage from 170 rudiments was used (five groups per determination).

Tissue	Total time in culture (days)	DNA content (μg/rudiment)	Hexosamine content of cartilage (μg/rudiment)	Tissue gain or loss per period of culture (μg/rudiment; mean values)	Hexosamine content of medium (μg/rudiment)	Total net synthesis (μg/rudiment; mean values)	Difference between treated and control (%)
Ex ovo	0		47.0±2.0				
Control culture	2	30.8±1.3	64.5±2.1	+17.5	9.6±1.32	+27.1	
	4	32.7±2.4	85.0±3.3	+20.5	4.3±2.06	+24.8	
	6	38.2±3.1	132.4±4.6	+47.4	9.7±2.8	+57.1	
Hyaluronidase-treated culture	2	27.4±3.3	15.8±0.9	−31.2	88.9±2.3	+57.7	+103.0
	4	30.4±1.4	52.9±2.3	+37.1	16.1±2.6	+53.2	+114.5
(Period of recovery)	6	34.4±2.1	105.3±3.6	+52.4	18.7±2.3	+71.1	+ 24.5

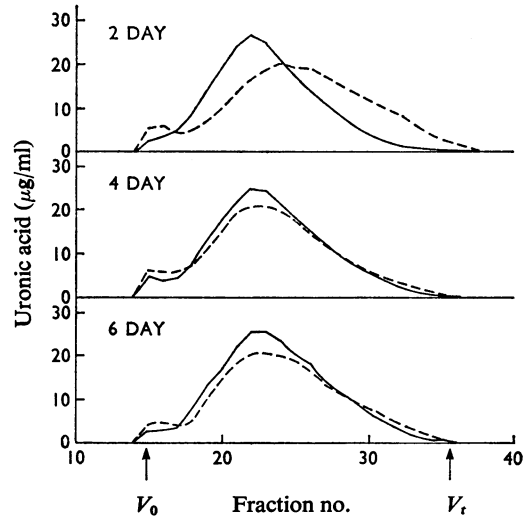


Fig. 3. *Elution of glycosaminoglycans from Sephadex G-200*

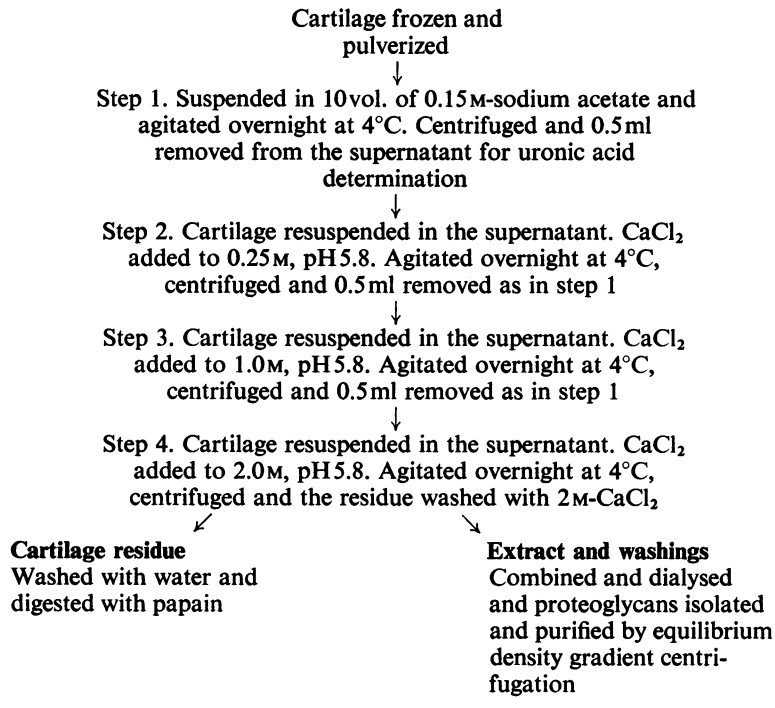
Samples of whole papain digests of control (——) and hyaluronidase-treated (– – – –) cartilage (series B) containing about 1 mg of uronic acid were applied to a column (120 cm × 1.1 cm) of Sephadex G-200 eluted with 0.2 M-sodium acetate, pH 6.8, with a hydrostatic pressure of 50 cm. The uronic acid content of each 2.2 ml fraction was determined. V_0 and V_t are described in Fig. 1.

Table 4. *Hyaluronidase activity in the media of treated tissue (series A)*

No activity was detectable in media from control cultures. n.d., Not detectable.

Period of culture (days)	Hyaluronidase activity (% of original)
Starting medium containing 500 μg of hyaluronidase/ml	100
0–2 days	46
First wash	2.3
Second wash	0.01
2–3 days	0.16
3–4 days	0.05
4–5 days	n.d.
5–6 days	n.d.

between these ratios in residues of control and treated tissue; this material was not characterized further and the galactosamine may not have been wholly due to proteoglycans left behind in the residue.



Scheme 1. *Flow diagram of the extraction of proteoglycans with a solution of increasing ionic strength (procedure II)*

Table 5. *Analysis of gel-chromatographic fractions of proteoglycans separated on Sepharose 2B (as shown in Fig. 1)*

The proteoglycans were extracted by procedure I from control and hyaluronidase-treated cartilage (series B).

	Time in culture (days)	Uronic acid extracted from cartilage (% of total)	Gel-chromatographic fractions	Uronic acid in fractions (% of total eluted)	Weight ratio uronic acid / protein	Molar ratio galactosamine / glucosamine
Control	2	86.1	E	37	0.88	10.7
			R	63	1.72	31.0
	4	84.8	E	35	0.74	10.4
			R	65	1.66	26.8
	6	85.8	E	35	0.78	8.2
			R	65	1.63	25.3
Hyaluronidase-treated	2	46.8	E	4		
			R_1	67	0.85	2.63
			R_2	29		
(Period of recovery)	4	49.2	E	9	0.48	3.8
			R_1	74	1.03	16.8
			R_2	17	0.36	10.1
	6	69.0	E	14	0.52	5.9
			R_1	77	1.32	21.7
			R_2	9	0.26	2.0

Table 6. *Analysis of gel-chromatographic fractions of proteoglycans separated on Sepharose 2B (as shown in Fig. 3)*

The proteoglycans were extracted by procedure II (see the text) from control and hyaluronidase-treated cartilage (series B).

	Time in culture (days)	Gel-chromatographic fractions	Uronic acid in fractions (% of total eluted)	Weight ratio uronic acid / protein	Molar ratio galactosamine / glucosamine
Control	2	E	41.0	1.02	11.8
		R_1	50.0	1.88	31.3
		R_2	9.0	1.10	26.8
	4	E	36.8	1.03	13.9
		R_1	54.4	1.92	51.6
		R_2	8.8	1.06	27.6
	6	E	40.7	1.06	11.8
		R_1	48.9	1.89	34.7
		R_2	10.3	1.18	26.8
Hyaluronidase-treated	2	E	3.6	0.39	1.74
		R_1	48.6	0.87	2.80
		R_2	47.8	0.47	2.04
(Period of recovery)	4	E	15.8	0.58	6.1
		R_1	53.0	1.06	13.5
		R_2	31.2	0.49	4.9
	6	E	27.6	0.69	8.3
		R_1	54.1	1.28	17.2
		R_2	18.3	0.61	6.7

Table 7. *Analysis of proteoglycans extracted by procedure II and of the resulting residues of control and hyaluronidase-treated cartilage (series B)*

	Time in culture (days)	Uronic acid extracted from cartilage (% of total)	Weight ratio uronic acid / protein	Molar ratios galactosamine / glucosamine	
				Extracted proteoglycans	Cartilage residue
Control	2	85.9	1.51	16.5	2.58
	4	84.9	1.51	15.1	2.64
	6	81.1	1.44	17.2	2.17
Hyaluronidase-treated	2	80.1	0.79	2.48	1.01
(Period of recovery)	4	75.1	0.96	10.1	3.42
	6	73.1	1.10	10.8	3.59

Discussion

The present investigation shows that proteoglycans synthesized after the removal of chondroitin sulphate by the action of hyaluronidase on the cartilaginous portions of embryo chick tibiae grown in organ culture, not only were of smaller molecular size immediately after treatment, but also differed in size and composition from those in control cartilage throughout a 4 day period of recovery, by which time a large proportion of the lost hexosamine had been replaced.

The present results extend the observations of Fitton-Jackson (1970*a*), in which whole tibial rudiments treated with hyaluronidase for 48h lost some 10% of their hydroxyproline, but 70% of their total hexosamine, the oligosaccharides produced by the action of hyaluronidase on chondroitin sulphate having diffused out of the tissue. At the same time there was a marked loss of metachromasia, particularly in the epiphysis, where it almost disappeared, compared with the cone region of the cartilage where some metachromasia persisted; such differences were

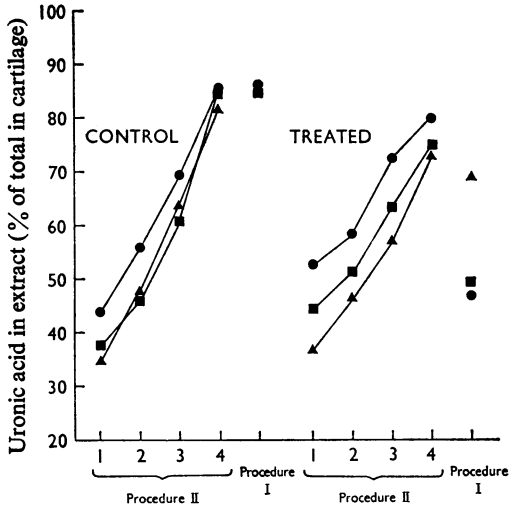


Fig. 4. *Proportion of the total proteoglycan in control and hyaluronidase-treated cartilage (series B) extracted at each of steps* 1–4 *of procedure II (see Scheme* 1)

The yields after 2 days (●), 4 days (■) and 6 days (▲) in culture are shown. Yields from procedure I (a single step) are shown for comparison.

probably due in part to regional variations in the distribution of intercellular constituents (Fitton-Jackson, 1960, 1968). In the present study, where the cartilage portions had been dissected from the tibiae and grown in direct contact with the medium, rather more hexosamine was lost from the tissue than when the cone region was protected by a collar of periosteal bone, as in whole rudiments grown under similar conditions.

The action of hyaluronidase caused a large amount of the proteoglycan to be lost from the cartilage so that only 25% of the hexosamine was left in the tissue compared with controls. At the same time there was a marked increase in the total synthesis of hexosamine, which continued for at least 4 days after removal of enzyme, during which time the hexosamine content of the tissue increased to 79% of the controls (Table 3). This represented an increase of 600% in the hexosamine content of the treated tissue during 4 days of recovery, as against 100% increase in controls. Hence most of the proteoglycans that were examined from treated cartilage during recovery were newly formed.

The proteoglycans in control cartilage changed little in composition during culture, and were the same as those present initially *in ovo*, which resembled those in mammalian cartilage of various sources (Tsiganos & Muir, 1969; Brandt & Muir, 1969, 1971; Tsiganos *et al.*, 1971). On the other hand, the proteoglycans in tissue after treatment with hyaluronidase were qualitatively different from those in control tissue. The preferential loss of chondroitin sulphate would explain why those extracted from cartilage immediately after hyaluronidase treatment were of smaller molecular size and contained a higher proportion of glucosamine and protein than proteoglycans extracted from control cartilage (Figs. 1 and 2). If some of these remained in the tissue during recovery they might account for some of the proteoglycans of smallest size (R_2, Figs. 1 and 2), which were of abnormally low uronic acid content, but this would not explain the observed differences in proteoglycans of large and medium size (E and R_1, Figs. 1 and 2), which had been newly synthesized during recovery. The fact that the chondroitin sulphate chains were of similar length in control and in treated cartilage recovering from the effects of hyaluronidase, not only confirms that proteoglycans in the latter were newly formed but that their altered composition was due not to the presence of shorter chondroitin sulphate chains but to fewer chains per molecule. This could have arisen either from a failure to glycosylate the core protein to the usual extent, at a time when depleted matrix is being replaced, or perhaps specific proteoglycans of a different kind containing less chondroitin sulphate and possibly more keratan sulphate may have been formed at this time. Alternatively, there may have been a change merely in the balance of the existing population of normal proteoglycans towards those of smaller size and lower chondroitin sulphate content. Minor fractions of such proteoglycans are present in normal pig laryngeal cartilage (Tsiganos *et al.*, 1971) and it is notable that in experiments *in vitro* the various proteoglycans in pig cartilage appear to be formed independently of each other (Hardingham & Muir, 1972). Whatever caused the change in the proteoglycans it was clear that as the content of hexosamine per mg dry wt. returned to the control value, the disturbance in the balance of the normal population of proteoglycans diminished.

The observed differences between proteoglycans extracted from control and treated cartilage may have been enhanced by a differential loss into the medium of those containing a high proportion of chondroitin sulphate. Fitton-Jackson (1970*a*) found that a considerable proportion of the material containing hexosamine formed during the period of recovery, and which contained intact chondroitin sulphate chains, had diffused into the medium; probably the depleted matrix was more permeable because of the diminished content of proteoglycans and thus allowed the macromolecules to diffuse into the medium. It seems unlikely, however, that differential diffusion into the medium would entirely

account for the qualitative differences between the proteoglycans extracted from control and treated cartilage, since at the end of the recovery period, when much of the depletion had been made good, qualitative differences still persisted. It is also unlikely that the differences between proteoglycans of control and treated cartilage extracted by procedure II resulted from the action of degradative enzymes during extraction because the proteoglycans extracted by both procedures from control cartilage were similar in size and composition (Tables 5 and 6; Figs. 1 and 2) and the ionic strength used in procedure I would probably have been high enough to stop the action of any degradative enzymes that may have been present.

Whether proteoglycan aggregates occur in embryo chick cartilage, as in adult mammalian cartilage (Sajdera & Hascall, 1969; Rosenberg *et al.*, 1970), is not known. In mammalian cartilage an increase in size with aggregation is accompanied by an increase in protein and keratan sulphate content; in contrast the proteoglycans in treated cartilage, although of higher protein and glucosamine content than controls, were of smaller size. Hence, disaggregation alone would not explain the observed changes. It has been reported (Hardingham & Muir, 1972) that in mammalian cartilage undissociated proteoglycans of intermediate size (similar to R_1, Fig. 2) were not able to form aggregates. Thus the diminished amount of largest proteoglycans (E, Fig. 2) present in recovering cartilage might be explained if hyaluronidase had affected the ability of proteoglycans to aggregate, and if most of the proteoglycans formed during recovery were incapable of aggregation.

The present results, and those of Bosmann (1968) and of Fitton-Jackson (1970*a*, 1972), show that in embryonic cartilage the matrix itself appears to exert some control over the synthetic processes of chondrocytes, since they are thus able to respond rapidly to the depletion of their matrix. The same response probably occurs in cartilage of young mammals since the intravenous injection of papain caused a loss of chondroitin sulphate from cartilage, which was rapidly replaced (Thomas, 1956; Spicer & Bryant, 1957; Tsaltas, 1958; Bryant *et al.*, 1958).

In recovering from acute exposure to a degradative enzyme, the proteoglycans formed by embryonic cartilage, being of different composition and smaller hydrodynamic size than those in unaffected cartilage, may function less efficiently and be more easily lost from the matrix. If chronic exposure of adult cartilage to degradative enzymes in pathological conditions led to similar qualitative changes in proteoglycans, a vicious circle might be established in which the loss of proteoglycans would stimulate cells to produce more of these macromolecules, which being qualitatively different might be more readily lost from the tissue; such a sequence of events might explain, in part, why degeneration of articular cartilage is usually progressive.

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