Biochem. J. (1980) **188**, 119–130
Printed in Great Britain

Control of Proteoglycan Synthesis

STUDIES ON THE ACTIVATION OF SYNTHESIS OBSERVED DURING CULTURE OF ARTICULAR CARTILAGES

John D. SANDY, Heather L. G. BROWN and Dennis A. LOWTHER
Department of Biochemistry, Monash University, Clayton, Vic. 3168, *Australia*

(*Received* 1 *October* 1979)

When slices of adult rabbit articular cartilage were incubated in culture medium, the rate of incorporation of [^{35}S]sulphate or [^{3}H]acetate into glycosaminoglycans increased 4–8-fold during the first 5 days of incubation. Similar changes in biosynthetic activity were observed during culture of adult bovine cartilage. The activation of synthesis was not serum-dependent, but appeared to be a result of the depletion of tissue proteoglycan that occurs under these incubation conditions [Sandy, Brown & Lowther (1978) *Biochim. Biophys. Acta* **543**, 536–544]. Thus, although complete activation was observed in serum-free medium, it was not observed if the cartilage was cultured inside dialysis tubing or in medium containing added proteoglycan subunit. The average molecular size of the proteoglycans synthesized by activated tissue was slightly larger than normal, as determined by chromatography on Sepharose CL-2B, and the average molecular size of the glycosaminoglycans synthesized by activated tissue was markedly increased over the normal. The increase in chain size was accompanied by an increase in the proportion of the chains degraded by chondroitinase ABC; these results are consistent with the preferential synthesis by activated chondrocytes of chondroitin sulphate-rich proteoglycans. The increase in glycosaminoglycan chain size was observed whether the chains were formed on endogenous core protein or on exogenous benzyl-β-D-xyloside. An approximate 4-fold activation in culture of glycosaminoglycan synthesis on protein core was accompanied by a 1.54-fold increase in the rate of incorporation of [^{3}H]serine into the chondroitin sulphate-linkage region of the proteoglycans. A 2.8-fold activation in culture of glycosaminoglycan synthesis on benzyl-β-D-xyloside was accompanied by a 1.7-fold increase in the rate of incorporation of [^{3}H]benzyl-β-D-xyloside into glycosaminoglycans. The activation of glycosaminoglycan synthesis was, however, accompanied by no detectable change in the activity of xylosyltransferase (EC 2.4.2.26) in cell-free extracts. These results are discussed in relation to current ideas on the control of proteoglycan synthesis in cartilage.

There is now a considerable body of evidence that indicates that the rate of synthesis of proteoglycan by chondrocytes is controlled by the extracellular concentration of cartilage macromolecules. Thus the removal of proteoglycan from cartilage matrix by treatment with papain (Bosmann, 1968), hyaluronidase (Hardingham *et al.*, 1972) or trypsin (Millroy & Poole, 1974) results in stimulation of proteoglycan synthesis and at least partial replacement of the lost proteoglycan. Also, the loss of cartilage proteoglycan in both human and experimental osteoarthrosis (Collins & McElligott, 1960; Mankin & Lippiello, 1970; Eronen *et al.*, 1978) is accompanied by increased rates of proteoglycan synthesis, assumed to be a cellular repair process. Further, the addition of hyaluronic acid (Wiebkin & Muir, 1973) or proteoglycan subunit (Handley & Lowther, 1977) to isolated chondrocytes in culture inhibits proteoglycan synthesis; moreover the inhibition by proteoglycan was reversed on its removal.

In studies on the maintenance of rabbit articular cartilage in organ culture, we observed that there was a spontaneous and extensive loss of proteoglycan from the tissue (Sandy *et al.*, 1978), and that this was accompanied by a marked increase in the rate of chondrocyte proteoglycan synthesis (Sandy

0306-3283/80/040119-12$01.50/1

et al., 1977). Activation of proteoglycan synthesis during culture of rabbit cartilage has also been observed by other workers (Lane & Brighton, 1974; Mitrovic *et al.*, 1978; Benya & Nimni, 1979); however, a detailed study of this phenomenon has not yet been reported.

In the present paper we describe experiments that indicate that activation in culture is a response of the chondrocytes to loss of cartilage proteoglycan from the tissue. This system has been studied to elucidate the mechanism by which chondrocytes respond to rapid change in the extracellular concentration of proteoglycan.

Experimental

Materials

Sodium [^{3}H]acetate and [^{3}H]serine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bio-Gel P4 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Chondroitinase ABC was prepared from *Proteus vulgaris* as previously described (Robinson & Dorfman, 1969). Collagenase (type I from *Clostridum histolyticum*) was from Worthington Biochemical Corp., NJ, U.S.A., and trypsin (bovine pancreas, type III) was from Sigma. Nonidet P40 was from BDH Chemicals, Poole, Dorset, U.K. Benzyl-β-D-xyloside and [^{3}H]benzyl-β-D-xyloside were prepared by Dr. H. C. Robinson of this laboratory (Robinson *et al.*, 1975). Sources of all other materials used have been previously described (Sandy *et al.*, 1978; Sandy, 1979).

Methods

Organ culture and radioactive-isotope incorporation. The conditions of organ culture of rabbit cartilage were as previously described (Sandy *et al.*, 1978). Cartilage slices were cultured in the medium described by Handley & Lowther (1977). Bovine cartilage was cultured under essentially the same conditions. Tissue was obtained from the metacarpalphalangeal joints of adult steers within 1 h of slaughter. The cartilage was freed of surrounding tissue, sliced finely and washed twice in medium before distribution into culture vials containing 150–200 mg wet wt./5 ml of medium. Incubation was at 37°C and the medium was renewed every 24 h. The procedures for incubations with radioactive isotopes and isolation of labelled glycosaminoglycans by papain digestion and DEAE-cellulose chromatography have been described (Handley & Lowther, 1976; Lowther *et al.*, 1978). Cartilage was removed from organ-culture medium and washed twice in Eagle's basal medium at 37°C and distributed into duplicate vials containing the same medium supplemented with 1 mM-glutamine and 0·5 mM-sodium acetate. After 30 min at 37°C, radioactive isotope was added and one vial was incubated for 2 h and the other for 4 h at 37°C under O_2/CO_2 (19:1). All tissue samples showed an essentially linear incorporation rate over this period of incubation. The amounts of each radioactive isotope and their specific radioactivities in cartilage incubations were as follows: [^{35}S]sulphate (25 μCi; 35.3 μCi/μmol); [^{3}H]acetate (180 μCi; 360 μCi/μmol); L-[^{3}H]serine (25 μCi; 34.7 μCi/μmol); [^{3}H]-benzyl-β-D-xyloside (250 μCi; 125 μCi/μmol). Each incorporation rate quoted in the Results section is the mean calculated from the 2 and 4 h determinations.

Organ culture with dialysis tubing. Dialysis tubing (Visking size 1–8/32") was obtained from Medicell International, London N1 1LX, U.K., and was washed by the following procedure before use in culture experiments. Pieces of tubing about 7 cm long were washed by 3 cycles of boiling and decanting in each of distilled water and 3% (v/v) acetic acid followed by soaking overnight in 10 mM-EDTA (sodium), pH 7·5, at 4°C. The pieces were then washed extensively in distilled water, autoclaved (15 min at 0.14 MPa) and washed in sterile distilled water before cartilage slices and medium were added. The use of surgical gloves in this procedure was avoided, since control experiments showed that material released from the gloves into the culture system was inhibitory to chondrocyte activity.

Determination of the percentage of chondroitin sulphate in isolated glycosaminoglycans. ^{35}S-labelled glycosaminoglycans for chondroitinase treatment were prepared from guanidine-extracted proteoglycans after removal of the extractant by dialysis against water for 16 h at 4°C. The proteoglycans were treated with 0.5 M-NaOH at 22–25°C for 24 h followed by addition of HCl to pH 8.0. The β-eliminated chains were treated with chondroitinase ABC (1 unit/mg of chondroitin sulphate) in 0.05 M-Tris/HCl (pH 8)/0.01 M-NaF for 16 h at 22–25°C (Robinson & Hopwood, 1973). One unit of chondroitinase ABC is that amount that produces an absorbance change at 230 nm of 1 in 1 min in 1 ml of 0.05 M-Tris/HCl (pH 8)/0.01 M-NaF containing 0.5 mg of chondroitin sulphate (Robinson & Dorfman, 1969). The digest was applied to a column (1.6 cm × 60 cm) of Bio-Gel P4 (200–400 mesh) eluted with 0.2 M-ammonium acetate, pH 6, at 17 ml/h. The column was calibrated with a chondroitinase digest of glycosaminoglycans prepared from purified proteoglycan subunit from bovine nasal cartilage. The elution position of the chondroitin sulphate disaccharides (100–115 ml) was determined by hexuronic acid and galactosamine analysis; this material was free of glucosamine. Most of

the glucosamine-containing material, probably keratan sulphate, was eluted as a peak at the void volume of the column (46–55 ml).

Isolation of chondroitin sulphate-linkage region. The method is based on that described by Robinson & Hopwood (1973) for isolation of the linkage region from bovine nasal cartilage proteoglycan. After incubations with [^{3}H]serine, the labelled glycosaminoglycans were isolated from cartilage by papain digestion and DEAE-cellulose chromatography (Handley & Lowther, 1976). The isolated product was desalted by dialysis against 0.05 M-Tris/HCl, pH 8, and digested with chondroitinase ABC as decribed above. The digest was applied to a column of Bio-Gel P4 (see above), and the linkage region was isolated as a sharp peak of labelled material recovered between 72 and 88 ml of eluate. The pooled fractions were freeze-dried and portions were hydrolysed in 4 M-HCl at 100°C for 8 h for hexosamine analysis or in 6 M-HCl at 110°C for 24 h *in vacuo* for amino acid analysis.

Extraction of radioactive proteoglycans. After incubation with radioactive isotope, cartilage was washed twice in fresh medium and extracted in 4 M-guanidine hydrochloride [containing 0.05 M-Tris/HCl buffer, pH 7.4, and the mixture of six proteinase inhibitors described by Pearson & Mason (1977)] for 24 h at 4°C, and the cartilage residue was removed by filtration on cottonwool.

Preparation of xyloside-initiated glycosaminoglycans from cartilage incubations. Cartilage was incubated under normal conditions (Lowther *et al.*, 1978) in medium containing 1 mM-benzyl-β-D-xyloside and [^{35}S]sulphate. The incubation was terminated by addition of an equal volume of 8 M-guanidine hydrochloride, and the whole was homogenized for 1 min in an ice bath with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) on speed setting 5. The homogenate was extracted for 24 h at 4°C and the cartilage residue was removed by filtration on cottonwool.

Other methods and analyses. The molecular weight of glycosaminoglycans formed in cartilage incubations was determined by chromatography on Sepharose CL-6B previously calibrated by the method of Hopwood & Robinson (1973).

Proteoglycan subunit added to cartilage in culture was prepared as previously described (Sandy, 1979) and sterilized by autoclaving (3 min at 0·14 MPa). DNA was determined by a fluorimetric method (Royce & Lowther, 1979). Galactosamine, glucosamine and amino acids were determined on a JOEL amino acid analyser as previously described (Sandy, 1979). Hexuronic acid was determined by a colorimetric method (Bitter & Muir, 1962) with glucuronolactone as standard. Protein was determined by a colorimetric method (Lowry *et al.*, 1951) with bovine serum albumin as standard.

Assay of UDP-xylose–protein xylosyltransferase (*EC* 2.4.2.26). The assay method was that described previously (Stoolmiller *et al.*, 1972). The substrate, Smith-degraded proteoglycan, was prepared by a modification (Sandy, 1979) of the original procedure (Baker *et al.*, 1972).

Extracts of whole cartilage were prepared at 0–4°C as follows: 0.25–0.30 g of bovine articular cartilage was frozen in liquid N_2 before homogenization in 2.5 ml of buffer [0.05 M-Mes buffer (4-morpholine-ethanesulphonic acid), pH 6.5, 0.2 M-KCl, 12 mM-$MgCl_2$, 3 mM-$MnCl_2$, 12.5 mM-KF] with the Polytron homogenizer. Homogenization was carried out for a total of 60 s on setting 5 and 20 s on setting 10. The homogenate was sonicated (25 × 1 s bursts; Branson sonifier, setting 5) and then centrifuged at 27000 *g* for 20 min. Portions of the clear supernatant were assayed for xylosyltransferase and protein.

In experiments where enzyme was assayed in isolated chondrocytes, the cells were prepared by treatment of cartilage slices (about 2 g) with trypsin and collagenase as described by Handley *et al.* (1975), except that the collagenase treatment was for 4 h at an enzyme concentration of 0.25% (w/v). The isolated cells (about 2×10^7/g of tissue) were suspended in 2.5 ml of homogenization buffer (see above) and disrupted by 20 × 1 s bursts on a Branson sonifier (setting 3) followed by three freeze–thaw cycles. The samples were then centrifuged at 100000 *g* for 1 h and the supernatants were assayed without further treatment. The pellet fractions were suspended for assay by gentle homogenization in assay buffer containing 0.5% Nonidet P40. All extracts containing xylosyltransferase were asayed in triplicate using 15, 30 and 60 μl of extract. In all cases there was a rectilinear relationship between reaction rate and enzyme concentration. One unit of enzyme activity is defined as that which catalyses the incorporation into trichloroacetic acid-insoluble material of 1 pmol of xylose/h under the conditions of the assay.

Results

Activation of proteoglycan synthesis in organ culture

When adult rabbit articular cartilage was incubated in organ culture, the rate of incorporation of [^{35}S]sulphate into glycosaminoglycans increased gradually by 4–8-fold during the first 5 days, and this high activity was maintained for up to 9 days (Fig. 1). In other incubations stimulated activity was maintained for 19 days. In similar experiments with adult bovine cartilage (Fig. 2) the increase in activity generally occurred in the first 24 h, and the overall activation was somewhat less than for the rabbit tissue. The shape of the activity curve between 0 and 24 h with bovine cartilage was determined in a

separate experiment with [^{35}S]sulphate as precursor. The biosynthetic activities determined with 1h labelling periods after 0, 6, 10 and 24h of culture were 14.0, 20.4, 26.5 and 61.0pmol of sulphate/h per μg of DNA respectively. It is therefore clear that at least 24h of culture is required for complete activation to occur.

The degree of activation was similar with [^{35}S]-sulphate and [^{3}H]acetate as precursors, showing that it involved an increased rate of incorporation of acetylhexosamine into glycosaminoglycan chains, and that it was not due to the synthesis by activated tissue of a highly sulphated form of the poly-saccharide. Results are expressed relative to the DNA content of the cartilage, which was essentially constant during the period of culture. The increased biosynthetic activity was therefore not due to cell proliferation.

The increase in activity was apparently not due to stimulation of chondrocytes by factors present in the serum component of the culture medium. The activation obtained was similar in the presence or absence of serum (Fig. 3). Further, the rate of sulphate incorporation in either fresh or cultured cartilage was not markedly altered by addition of 20% (v/v) foetal calf serum to short-term (4h) incubations.

Culture of rabbit articular cartilage under these conditions is accompanied by the loss of up to 60% of the proteoglycan from the tissue into the medium in the first 4 days (Sandy *et al.*, 1978). In recent experiments similar results were obtained with bovine tissue, although the rate of loss was somewhat lower, with about 10% release in 1 day and about 35% in 4 days. These findings, together with

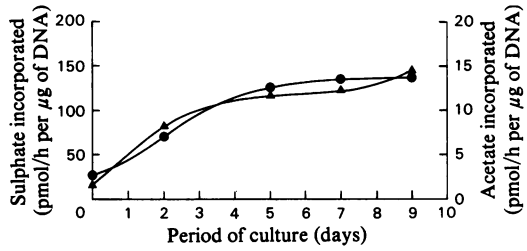

Fig. 1. *Effect of organ culture on the rate of proteoglycan synthesis in rabbit articular cartilage*
Rabbit articular cartilage (50–100mg wet wt.) was maintained in organ culture, and at various periods the rate of incorporation of [^{35}S]sulphate (●) and [^{3}H]acetate (▲) into glycosaminoglycans was determined. Radioactive glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography (see under 'Methods'). Each result is the mean of a 2 and 4h incorporation rate.

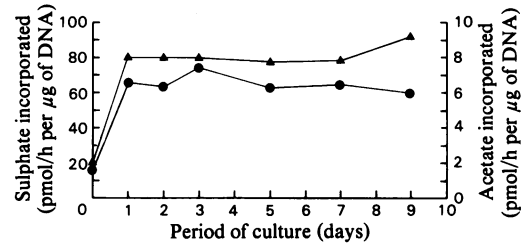

Fig. 2. *Effect of organ culture on the rate of proteoglycan synthesis in bovine articular cartilage*
Bovine articular cartilage (150–200mg wet wt.) was maintained in organ culture, and at various periods the rate of incorporation of [^{35}S]sulphate (●) and [^{3}H]acetate (▲) into glycosaminoglycans was determined. Radioactive glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography (see under 'Methods'). Each result is the mean of a 2 and 4h incorporation rate.

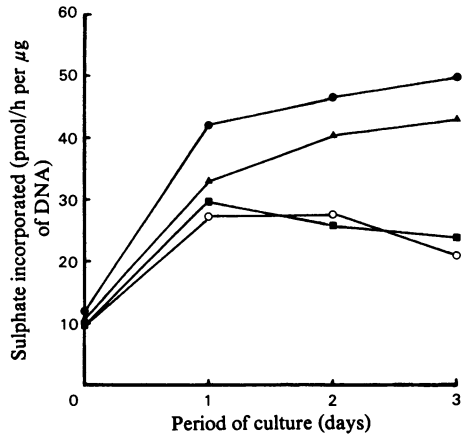

Fig. 3. *Effect of culture conditions on the activation of proteoglycan synthesis observed in bovine cartilage*
Bovine articular cartilage was maintained in organ culture and after 0, 1, 2 and 3 days the rate of incorporation of [^{35}S]sulphate into glycosaminoglycan was determined (see under 'Methods'). ^{35}S-labelled glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography (see under 'Methods'). Changes to the standard conditions of culture and the number of separate experiments done under each set of conditions (values in square brackets) were as follows: ●, no change [21]; ▲, culture in the absence of serum [3]; ■, culture in the presence of proteoglycan subunit at 10mg/ml [1]; O, culture with the tissue inside dialysis tubing [3]. Each point shown is the mean result calculated from all the data obtained under such conditions.

the known inhibitory effect of high concentrations of extracellular proteoglycan on chondrocyte biosynthetic activity (Handley & Lowther, 1977), indicated that activation in culture might be a direct result of the loss of tissue proteoglycan.

Experiments were done under conditions designed to interfere with the normal process of proteoglycan loss from the tissue during culture. Bovine cartilage was cultured under normal conditions, except that the tissue (about 75 mg wet wt.) was placed in 0.1 ml of medium inside dialysis tubing before incubation. There was no detectable release of hexuronate-positive material from the dialysis bag, and the activation process was markedly inhibited (Fig. 3). This inhibition was not due to inhibitory material released from the dialysis tubing in culture, since in control experiments where cartilage was cultured outside the tubing the normal complete activation was observed. In another set of experiments bovine cartilage was cultured with proteoglycan subunit added to the normal culture medium. The activity observed relative to the control, over 3 days of culture, was markedly inhibited by the presence of proteoglycan at 10 mg/ml (Fig. 3).

Characterization of proteoglycans synthesized by activated cartilage

The molecular size distribution of proteoglycans synthesized by normal and activated cartilage was determined by gel filtration of extracted proteoglycans on Sepharose CL-2B under dissociative conditions. For both rabbit cartilage (Fig. 4) and bovine cartilage (Fig. 5) the proteoglycans synthesized by activated tissue appeared slightly larger than the normal, as shown by a slight shift in the elution profiles towards the void volume of the column.

The molecular size distribution of the glycosaminoglycans was investigated by gel chromatography on Sepharose CL-6B. With rabbit cartilage, chains formed in activated tissue were much larger than normal (Fig. 6), as shown by a marked shift in the elution profile towards the void volume. Thus the average molecular weight of the glycosaminoglycans formed increased from 14000 to 32000 on activation in culture. Similarly, with bovine tissue (Fig. 7) the chains formed by cultured cartilage were markedly larger than normal; the molecular weight increased from an average of 16000 to 28000 on activation.

The [^{35}S]sulphate-labelled glycosaminoglycans synthesized by normal and activated rabbit cartilage during a 4h labelling period were analysed by digestion with chondroitinase ABC and chromatography on Bio-Gel P4 (see under 'Methods'). Since dermatan sulphate is a very minor component of articular cartilage, the percentage of the glycos-

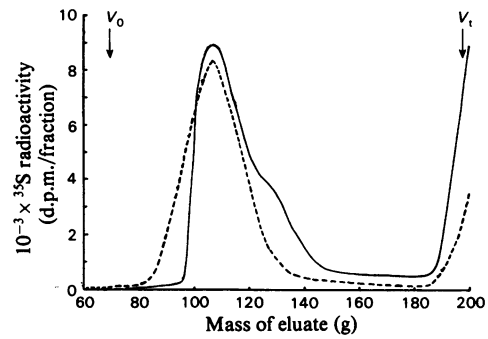

Fig. 4. *Elution from Sepharose CL-2B of proteoglycans synthesized by normal and activated rabbit cartilage*
Rabbit articular cartilage was placed in organ culture, and, after 0 and 8 days of culture, portions of tissue were labelled by incubation for 4h with [^{35}S]sulphate. Proteoglycans formed in normal (——) and activated (----) cartilage were extracted with 4M-guanidine hydrochloride (see under 'Methods') and samples, containing about 60000 d.p.m. of ^{35}S-labelled proteoglycan, were applied to a column (1.9cm × 65cm) of Sepharose CL-2B, which was eluted at 12ml/h with 2M-guanidine hydrochloride (containing 0.5M-sodium acetete, pH6.8) at 22–25°C. Fraction sizes (about 4g) were determined by mass. Recovery of radioactivity from the column was greater than 87% in each case. Abbreviations: V_0, void volume; V_t, total volume.

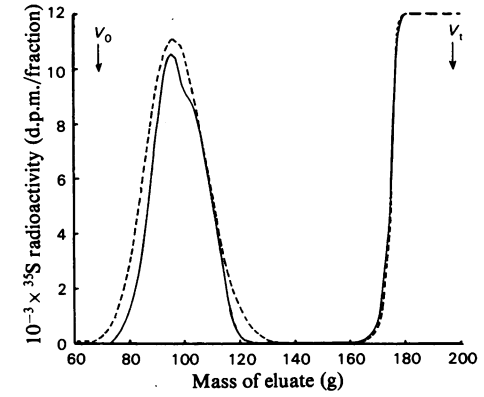

Fig. 5. *Elution from Sepharose CL-2B of proteoglycans synthesized by normal and activated bovine cartilage*
Bovine articular cartilage was placed in organ culture, and after 0 and 3 days of culture portions of the tissue were labelled by incubation for 4h with [^{35}S]sulphate. Proteoglycans formed in normal (——) and activated (----) cartilage were extracted with 4M-guanidine hydrochloride (see under 'Methods') and samples, containing about 60000d.p.m. of ^{35}S-labelled proteoglycan were fractionated on the Sepharose CL-2B column system described in the legend to Fig. 4. Recovery of radioactivity was greater than 83% in each case.

aminoglycans digested by chondroitinase ABC is a close approximation to the chondroitin sulphate content. In three such culture experiments chondroitin sulphate accounted for 75–80% of the radioactive glycosaminoglycan formed in normal cartilage, whereas it accounted for 93–96% of that formed in activated cartilage.

Mechanism of activation of proteoglycan synthesis

With rabbit articular cartilage, incubation in the presence of 1 mM-benzyl-β-D-xyloside results in an approximate 1.5–2.0-fold increase in the rate of sulphate incorporation, and more than 85% of the glycosaminoglycans formed are initiated by the xyloside. When cartilage was incubated in the presence of 1 mM-benzyl-β-D-xyloside and [^{35}S]-sulphate and then extracted with 4 M-guanidine hydrochloride, the extract contained more than 95% of the ^{35}S-labelled glycosaminoglycans. When this extract was fractionated on Sepharose CL-6B (Fig. 8), more than 90% of the ^{35}S-labelled glycosaminoglycans were recovered as a broad peak in the included volume of the column, which is consistent with the elution behaviour of free xyloside-initiated chains. A small amount

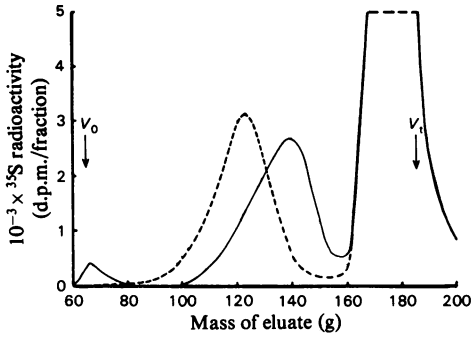

Fig. 6. *Elution from Sepharose CL-6B of glycosaminoglycans synthesized by normal and activated rabbit cartilage*

Rabbit articular cartilage was placed in organ culture, and after 0 and 8 days of culture portions of the tissue were labelled by incubation for 4 h with [^{35}S]sulphate. Glycosaminoglycans formed in normal (——) and activated (----) cartilage were prepared by papain digestion of the total incubation, and samples, containing about 30000 d.p.m. of ^{35}S-labelled glycosaminoglycan, were applied to a column (80 cm × 1.5 cm) of Sepharose CL-6B which was eluted at 12 ml/h and 22–25°C with 4 M-guanidine hydrochloride (containing 50 mM-Tris/HCl, pH 8.2). Fraction sizes (about 4 g) were determined by mass. Recovery of radioactivity was greater than 93% in each case, Abbreviations: V_0, void volume; V_t, total volume.

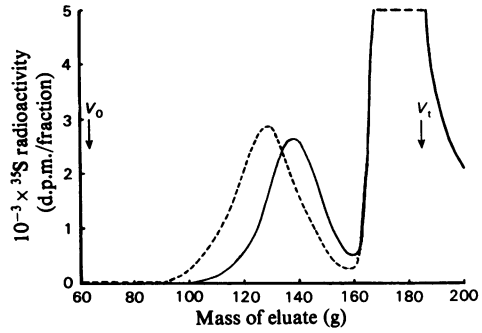

Fig. 7. *Elution from Sepharose CL-6B of glycosaminoglycans synthesized by normal and activated bovine cartilage*

Bovine articular cartilage was placed in organ culture, and, after 0 and 3 days of culture, portions of the tissue were labelled by incubation for 4 h with [^{35}S]sulphate. Glycosaminoglycans formed in normal (——) and activated (----) cartilage were prepared by papain digestion of the total incubation, and samples, containing about 30000 d.p.m. of ^{35}S-labelled glycosaminoglycan, were fractionated on the Sepharose CL-6B column system described in the legend to Fig. 6. Recovery of radioactivity was greater than 90% in each case.

Table 1 *Effect of benzyl-β-D-xyloside on glycosaminoglycan synthesis in articular cartilages*

Rabbit and bovine cartilage slices were cultured under standard conditions (see under 'Methods'), and after the periods shown the rates of incorporation of [^{35}S]sulphate into glycosaminoglycans were determined in the absence and presence of 1 mM-benzyl-β-D-xyloside. ^{35}S-labelled glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography (see under 'Methods'). Each result is the mean of a 2 and 4 h incorporation rate.

Cartilage	Period of culture (days)	Biosynthetic rate (pmol of SO_4 incorporated/h per μg of DNA)	
		No addition	+ 1 mM-Benzyl-β-D-xyloside
Rabbit	0	13.3	22.1
	8	61.2	83.1
Bovine	0	16.2	39.5
	3	62.1	110

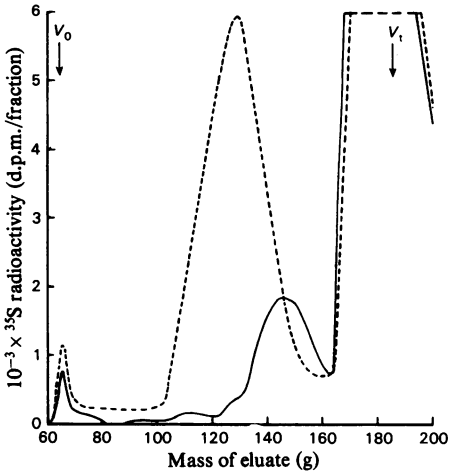

Fig. 8. *Elution from Sepharose CL-6B of xyloside-initiated glycosaminoglycans synthesized by normal and activated rabbit cartilage*

Rabbit articular cartilage was placed in organ culture and after 0 and 8 days of culture, samples of the tissue were incubated for 4h with [^{35}S]sulphate in the presence of 1mM-benzyl-β-D-xyloside (see under 'Methods'). The ^{35}S-labelled glycosaminoglycans formed in normal (——) and activated (----) cartilage were extracted from homogenized tissue in 4M-guanidine hydrochloride (see under 'Methods') and samples, containing 10000–30000d.p.m. of ^{35}S-labelled glycosaminoglycans, were fractionated on the Sepharose CL-6B column system described in the legend to Fig. 6. Recovery of radioactivity was greater than 87% in each case.

of radioactive product, probably protein-bound glycosaminoglycan, was eluted at the void volume. The free glycosaminoglycans recovered in the included volume by this method were not core-protein-initiated chains released from proteoglycan during isolation, since incubations done in the absence of xyloside and extracted in an identical manner yielded profiles with all of the material eluted at the void-volume and no radioactivity in the free glycosaminoglycan position.

The effect of xyloside on the activity exhibited by normal and activated cartilage from both sources is shown in Table 1. The degree of culture activation observed in these experiments, with endogenous core protein as substrate, was 4.60-fold for rabbit cartilage and 3.83-fold for bovine tissue. With benzyl-β-D-xyloside as substrate, however, the degree of activation was significantly less, being 3.76-fold and 2.81-fold respectively.

The increase in the size of chains formed on endogenous core protein on activation of rabbit cartilage (Fig. 6) was also observed when chains were formed on exogenous benzyl-β-D-xyloside (Fig. 8). In this case, the average molecular weight of the xyloside-initiated chains formed by normal tissue, about 7000, increased very markedly to about 26000 on culture activation.

The results described above show that the increase in biosynthetic rate was accompanied by the synthesis of longer than normal chondroitin sulphate chains. This increase occurred whether the chains were formed on core protein or on benzyl-β-D-xyloside. The increase in the size of protein-initiated chains was, however, only about 2-fold (Figs. 6 and 7), and this could not account entirely for the increase in the rate of radioactive-isotope incorporation, which was generally 4–8-fold (Figs. 1 and 2). This indicated that not only the size of chains but the rate of chain initiation must alter on activation. To confirm this, a number of experiments were carried out with bovine articular cartilage.

Firstly, the rate of incorporation of [^{3}H]serine into the chondroitin sulphate-linkage region was determined in normal and activated tissue. Amino acid analysis of purified linkage region (see under 'Methods') showed that it contained 29.7% serine, 16.1% glycine, 9.6% glutamic acid, 9.2% leucine, 7.9% proline and 7.7% alanine. Other amino acids were present in smaller amounts. Glucosamine was absent and the galactosamine/serine ratio was 1.31:1. These results are consistent with the localization of chondroitin sulphate on the linear polysaccharide attachment region of the core protein (Rosenberg *et al.*, 1976), and also with the proposed structure of the linkage oligosaccharide isolated by this procedure from bovine nasal-cartilage proteoglycan (Robinson & Hopwood, 1973). Further, radioactive isotope analysis of the column effluent from the amino acid analyser established that the radioactivity of the isolated linkage region samples was present exclusively in the serine residues.

In two culture experiments (Table 2) a 3.4-fold and 4.4-fold increase in sulphate incorporation into glycosaminoglycan was accompanied by a 1.52-fold and 1.55-fold increase in serine incorporation respectively. The increased rate of synthesis of linkage region is direct evidence that the activation mechanism involves an increase in the rate of chain initiation by xylosyltransferase. As a further check that the number of chains formed per unit time was increased on activation, the rate of incorporation of [^{3}H]benzyl-β-D-xyloside into glycosaminoglycans in normal and activated tissue was also determined. In this case (Table 3) a 2.9-fold and 2.7-fold increase in sulphate incorporation into xyloside-initiated chains was accompanied by a 1.6-fold and 1.8-fold increase respectively in the rate of [^{3}H]benzyl-β-D-xyloside incorporation into these chains. These results substantiate those obtained with [^{3}H]serine

Table 2. *Effect of culture of bovine cartilage on the rate of incorporation of [^{3}H]serine into the chondroitin sulphate-linkage region*

Bovine cartilage slices were cultured under standard conditions (see under 'Methods'), and after the period shown the cartilage from each culture was divided for determination of the rate of incorporation of [^{35}S]sulphate into glycosaminoglycans and of [^{3}H]serine into chondroitin sulphate-linkage region. ^{35}S-labelled glycosaminoglycans and ^{3}H-labelled glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography, and ^{3}H-labelled linkage region was isolated from ^{3}H-labelled glycosaminoglycans by chondroitinase digestion and Bio-Gel P4 chromatography (see under 'Methods'). Each result is the mean of a 2 and 4 h incorporation rate.

		Biosynthetic rate	
Expt.	Period of culture (days)	Glycosaminoglycan synthesis (pmol of SO_4 incorporated/h per μg of DNA)	Linkage-region synthesis (pmol of serine incorporated/h per μg of DNA)
1	0	24	0.88
	3	82	1.34
2	0	18	0.64
	3	80	0.99

Table 3. *Effect of culture of bovine cartilage on the rate of incorporation of [^{3}H]benzyl-β-D-xyloside into glycosaminoglycans*

Bovine cartilage slices were cultured under standard conditions (see under 'Methods'), and after the periods shown the cartilage from each culture was divided for determination of (i) the rate of incorporation of [^{35}S]sulphate into glycosaminoglycans in the presence of 1 mM-benzyl-β-D-xyloside and (ii) the rate of incorporation of [^{3}H]benzyl-β-D-xyloside into glycosaminoglycans at a benzyl-β-D-xyloside concentration of 1 mM. ^{35}S-labelled glycosaminoglycans and ^{3}H-labelled glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography (see under 'Methods'). Each result is the mean of a 2 and 4 h incorporation rate.

		Xyloside-initiated glycosaminoglycan synthesis	
Expt.	Period of culture (days)	(pmol of SO_4 incorporated/h per μg of DNA)	(pmol of benzyl-xyloside incorporated/h per μg of DNA)
1	0	39	4.9
	3	112	7.8
2	0	40	3.8
	3	107	6.9

incorporation and confirm that the activation involves not only an increase in the length of the glycosaminoglycans (Figs. 6 and 7) but also an increase in the number of chains formed per unit time.

It should be noted that the data described in Table 3 are also an indirect measure of the size of the chondroitin sulphate chains formed in normal and activated cartilage. Thus the ratio of sulphate incorporation to xyloside incorporation, given that these are incorporated solely into glycosaminoglycans, is an approximate measure of the number of sulphate groups/xyloside-initiated chain. The results obtained in this way, assuming one sulphate group/disaccharide, indicate that in normal tissue the xyloside-initiated chains are of about 5000 mol. wt., whereas in stimulated cartilage they are about 8000 mol.wt. These calculated values therefore support the finding (Fig. 8) that the size of xyloside-initiated chains increases on activation of rabbit articular cartilage.

Studies on xylosyltransferase

The xylosyltransferase activity of a soluble fraction derived from homogenates of normal and cultured bovine cartilage was determined. Whereas glycosaminoglycan synthesis was activated by up to 8-fold during the culture period, the activity of xylosyltransferase remained essentially constant at about 25 units/mg of protein (Fig. 9). In another culture experiment the chondrocytes were isolated from cartilage slices by proteolytic digestion before enzyme extraction (see under 'Methods'). In this case activity was demonstrated in both the soluble and particulate fractions of the isolated cells (Table 4). However, an increase in the rate of glycos-

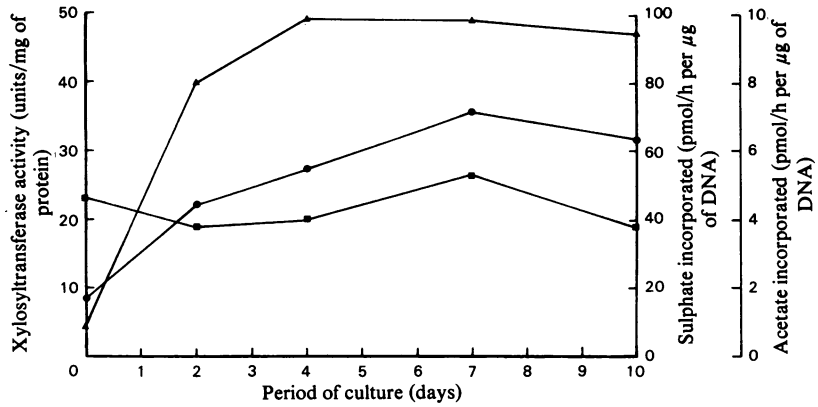

Fig. 9. *Effect of organ culture on the rate of proteoglycan synthesis and the activity of xylosyltransferase in bovine articular cartilage*

Bovine articular cartilage was maintained in organ culture, and at various periods the rate of incorporation of [^{35}S]sulphate (●) and [^{3}H]acetate (▲) into glycosaminoglycans was determined. Radioactive glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography (see under 'Methods') and each result shown is the mean of a 2h and 4h incorporation rate. The activity of xylosyltransferase (■) was determined in cell-free extracts (see under 'Methods') of cartilage culture in parallel incubations. Each xylosyltransferase result is the mean of the activity obtained with 15μl, 30μl and 60μl of enzyme extract.

Table 4. *Effect of culture on xylosyltransferase activity in chondrocytes isolated from bovine cartilage*

Bovine cartilage slices were cultured under standard conditions (see under 'Methods'), and after the periods shown the cartilage from each culture was divided for determinations of (i) the rate of incorporation of [^{35}S]sulphate into glycosaminoglycans and (ii) the activity of xylosyltransferase in the soluble and particulate fractions of chondrocytes isolated from the cartilage (see under 'Methods'). ^{35}S-labelled glycosaminoglycans were isolated from cartilage plus medium by papain digestion and DEAE-cellulose chromatography (see under 'Methods') and each result is the mean of a 2 and 4h incorporation rate. Each xylosyltransferase result is the mean of the activity obtained with 15, 30 and 60μl of enzyme extract.

Period of culture (days)	Glycosaminoglycan synthesis (pmol of SO_4 incorporated/h per μg of DNA)	Xylosyltransferase activity (units/mg of protein)	
		Soluble fraction	Particulate fraction
0	35.7	60.1	81.2
3	76.1	50.9	77.1

aminoglycan synthesis in the whole tissue from 35.7 to 76.1pmol of sulphate/h per μg of DNA was accompanied by no marked changes in the specific activity of xylosyltransferase in either the soluble fraction or the particulate fraction of the isolated chondrocytes.

Discussion

One of the basic techniques used in the study of glycosaminoglycan and proteoglycan metabolism in articular cartilage is the incorporation of radioactive precursors into cartilage slices *in vitro*. Such radioactive-isotope studies, generally performed with [^{35}S]sulphate, have been used in the analysis of proteoglycan metabolism in both normal and pathological tissues.

Metabolic studies from a number of laboratories have shown that radioactive-isotope incorporation by cartilage is very sensitive to variation in the methods of tissue preparation (Hardingham & Muir, 1970) and conditions of incubation (Maroudas & Evans, 1974; Lemperg *et al.*, 1977). In particular, three reports (Lane & Brighton, 1974; Mitrovic *et al.*, 1978; Benya & Nimni, 1979) have shown that proteoglycan synthesis in rabbit cartilage increases markedly during prolonged incubation of the tissue in nutrient medium containing serum. We have also observed (Sandy *et al.*, 1977) that glycosaminoglycan synthesis in rabbit cartilage is markedly stimulated during organ culture and that such cartilage undergoes extensive loss of high-molecular-weight tissue proteoglycan during the culture period (Sandy *et al.*, 1978).

The results in the present paper indicate that activation of proteoglycan synthesis in culture is a chondrocyte response to loss of extracellular proteoglycan. This is suggested firstly by the finding that the activation process can be markedly inhibited by incubation of the cartilage under conditions expected to interfere with the normal loss of tissue proteoglycan. Thus cartilage cultured inside dialysis tubing or in the presence of a high concentration of added proteoglycan subunit does not exhibit the normal degree of activation (Fig. 3). Since proteoglycan is not lost from the dialysis bag during culture, the effect of this procedure is to decrease the possible maximum dilution of tissue proteoglycan from about 25-fold in the normal culture system to only about 2-fold inside the dialysis bag. It is not known whether the inhibiting effect of added proteoglycan is due to limitation of the loss of endogenous proteoglycan from the cartilage or replacement of that which is lost. In either case the results support the idea that activation in culture is a chondrocyte response to loss of extracellular proteoglycan.

The finding that chondrocytes activated in culture produce chondroitin sulphate-rich proteoglycan is also consistent with a cellular response to matrix depletion. Thus the formation of such proteoglycans has been widely reported to occur in both experimental and human osteoarthrosis (McDevitt *et al.*, 1973; Mankin, 1973; Bayliss & Ali, 1978), apparently in response to depletion of cartilage proteoglycan after degradative changes in the joint. It is also significant that chondrocytes with high biosynthetic activity present in immature pig cartilage (Simunek & Muir, 1972) and human cartilage (Benmaman *et al.*, 1969) produce proteoglycans with a markedly higher content of chondroitin sulphate than the proteoglycans formed at a lower rate in mature tissues. If the activation observed in the present work is indeed a chondrocyte response to proteoglycan loss, then it is clear that only slight changes in the total tissue content of proteoglycans are required for marked stimulation of cellular activity. Thus in cultured bovine cartilage the activation observed in the first 24h (Fig. 2) is accompanied by a loss of only about 10% of the tissue proteoglycan into the medium. It may be, however, that this loss is confined to the pericellular regions of the tissue, thereby producing a very marked change in proteoglycan concentration at or near the chondrocyte surface.

It seems unlikely that the activation is due to an alteration during culture of such factors as oxygen tension (Lane *et al.*, 1977), temperature (Hardingham & Muir, 1970) or nutrient supply (Audhya & Gibson, 1976), all of which have been shown to influence either the rate of synthesis or the type of glycosaminoglycan formed in articular cartilage. Thus the effect of such factors should not be influenced by culture of the tissue inside dialysis tubing, or the addition of exogenous proteoglycan, both of which were found to inhibit the activation process markedly (Fig. 3). The results described also eliminate the possibility that the activation is due to a stimulatory effect of serum components, such as observed with embryonic-chick cartilages (Nevo *et al.*, 1978).

Nature of the chondrocyte response

Although it is accepted that chondrocytes respond to alterations in the extracellular proteoglycan concentration by changing their rate of synthesis, the mechanism by which the cells achieve this change in metabolic state is unclear.

In the early work on proteoglycan depletion in embryonic-chick tibiae by hyaluronidase treatment (Bosmann, 1968; Fitton-Jackson, 1970) a stimulation in synthetic rate was noted, but the nature of the replacement proteoglycans was not described in detail. Later (Hardingham *et al.*, 1972) it was shown that embryonic-chick chondrocytes respond to such depletion by the rapid synthesis of proteoglycans of smaller than normal size, apparently as the result of a low degreee of substitution of the protein core with chondroitin sulphate chains. In contrast, studies on serum-stimulated proteoglycan synthesis in embryonic-chick cartilage indicate that the proteoglycan formed under these conditions has the normal complement of chondroitin sulphate chains per protein core (Gibson *et al.*, 1977).

In the present work it has been shown that activated chondrocytes in both rabbit and bovine cartilage also form structurally abnormal proteoglycans. Thus the size of the proteoglycan subunit formed by stimulated cells appears slightly larger than normal on Sepharose CL-2B filtration (Figs. 4 and 5). This increase in subunit size can be at least partly explained by the concomitant increase in the average molecular weight of the component glycosaminoglycans (Figs. 6 and 7). The increase in chain size, which for both tissues was about 2-fold, was, however, insufficient to account entirely for the observed increase in the rate of glycosaminoglycan synthesis, which was generally greater than 4-fold. This suggested that the activation must also involve an increase in the number of chains formed per unit time. To confirm this we determined the rate of incorporation of [^{3}H]serine into the isolated chondroitin sulphate-linkage region and found (Table 2) that the number of chains formed per unit time did indeed increase during culture by about 1.6-fold. If it can be assumed that only specific serine residues of the core protein are substituted with chondroitin sulphate (Baker *et al.*, 1972), then it appears that the rate of core-protein synthesis must also increase by 1.6-fold during the culture period. This conclusion is

also supported by the finding that the size of the proteoglycan formed under stimulation appears to be only slightly larger than normal. Thus, if the increased synthesis of linkage region was due instead to oversubstitution of core-protein serine residues with chondroitin sulphate chains about twice the normal size, then the size of the proteoglycan subunit expected would be much larger than that observed (Figs. 4 and 5).

With both rabbit and bovine cartilage the degree of activation demonstrable with xyloside as substrate was always less than that with core protein (Table 1). This implies that the activation process involves a greater increase in xylosyl-core-protein synthesis than in overall chain elongation and sulphation activity. That is, the increase in xylosyl-core-protein formation has apparently not been accompanied by as great an increase in chain-synthesizing activity, a finding that suggests that these two sections of the biosynthetic sequence are independently controlled.

In order to confirm that the rate of chain initiation had increased during culture, we also determined the rate of incorporation of [^{3}H]benzyl-β-D-xyloside into glycosaminoglycans in normal and activated cartilage (Table 3). Incubations with this substrate determine the rate of synthesis of linkage region and chondroitin sulphate by all the chondroitin sulphate glycosyltransferases except xylosyltransferase. We found, in close agreement with the results for [^{3}H]serine incorporation, that the rate of chain initiation had increased by about 1.6–1.8-fold during culture. The finding that [^{3}H]benzyl-β-D-xyloside is incorporated into glycosaminoglycans at about 10 times the rate of [^{3}H]serine is consistent with the finding (Robinson *et al.*, 1975) that xyloside in incubations results in the rapid formation of a large number of small chains, and also that xyloside, unlike serine, has no known metabolic fate other than glycosaminoglycan synthesis. It is of interest that the present work is apparently the first detailed account of [^{3}H]serine incorporation into an isolated and characterized linkage region in mammalian cartilage.

Xylosyltransferase is the first enzyme specific to chondroitin sulphate synthesis, and studies with embryonic-chick cartilage (Schwartz & Rodén, 1974; Schwartz, 1976) have indicated that the transfer of xylose to serine in the core protein may be the rate-determining step in chain synthesis. As a result of our finding that the activation of chondroitin sulphate synthesis in culture involves an increased rate of chain initiation (Tables 2 and 3), it seemed likely that it might also be accompanied by an increased activity of xylosyltransferase in cell-free extracts.

However, the assay of this enzyme in normal and activated cartilage (Fig. 9, Table 4) has shown that its activity apparently remains constant under conditions where the rate of chain initiation is increased by perhaps 1.6–1.8-fold. This may indicate that xylosyltransferase is not of critical importance in the control of proteoglycan synthesis in articular cartilages, although studies with embryonic-chick chondrocytes (Schwartz, 1976) have demonstrated that the enzyme has a short half-life, a property that is generally indicative of a regulatory role. Alternatively, the activity of xylosyltransferase may be controlled not by induction of its synthesis but by interaction of existing enzyme with other glycosyltransferases or membrane components (Schwartz, 1974). If xylosyltransferase were activated by some specific interaction, then this may be reversed by the preparation of the enzyme in a soluble form for assay.

This work has also demonstrated that mammalian chondrocytes have the capacity to alter the normal process of chondroitin sulphate synthesis rapidly in order to produce abnormally long chains. It may involve the synthesis *de novo* of the elongation enzymes glucuronosyltransferase II and *N*-acetylgalactosaminyltransferase, or perhaps activation of existing membrane-bound enzyme. Whatever the nature of this change, it is one that apparently does not require the presence of a core-protein-bound product for its expression. Thus an increase in the chain size of chondroitin sulphate was observed on activation, even when the chains were formed on exogenous benzyl-β-D-xyloside (Fig. 8). This would indicate that chain size is perhaps controlled by the prevailing activity, or distribution on the membrane, of the elongation enzymes rather than, as has been suggested, by some specific interaction between core protein and endoplasmic reticulum.

In summary, the data presented indicate that chondrocytes of adult mammalian cartilages have the capacity to respond quickly to rapid loss of extracellular proteoglycan, by an increase in biosynthetic rate. This increase apparently involves a de-repression of core-protein synthesis and the accelerated synthesis of chondroitin sulphate-rich proteoglycans with abnormally long glycosaminoglycan chains. This kind of chondrocyte response apparently occurs in osteoarthrosis, although there is no evidence for similar changes in acute inflammatory joint diseases such as rheumatoid arthritis. Thus joint inflammation in experimental arthritis is accompanied by a marked inhibition of chondrocyte biosynthetic activity (Lowther *et al.*, 1978).

We thank Dr. H. Clem. Robinson for the generous donation of xylosides and for helpful discussion. This work was supported by The Australian Research Grants Committee (grant no. 74-15501 R).

References

Audhya, T. K. & Gibson, K. D. (1976) *Biochim. Biophys. Acta* **437**, 364–376

Baker, J. R., Rodén, L. & Stoolmiller, A. C. (1972) *J. Biol. Chem.* **247**, 3831–3847

Bayliss, M. T. & Ali, S. Y. (1978) *Biochem. J.* **169**, 123–132

Benmaman, J. D., Ludoweig, J. J. & Anderson, C. E. (1969) *Clin. Biochem.* **2**, 461–464

Benya, P. D. & Nimni, M. E. (1979) *Arch. Biochem. Biophys.* **192**, 327–335

Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334

Bosmann, H. B. (1968) *Proc. R. Soc. London Ser. B* **169**, 399–425

Collins, D. H. & McElligott, T. F. (1960) *Ann. Rheum. Dis.* **19**, 318–330

Eronen, I., Videman, T., Friman, C. & Michelsson, J. E. (1978) *Acta. Orthop. Scand.* **49**, 329–334

Fitton-Jackson, S. (1970) *Proc. R. Soc. London Ser. B* **175**, 405–453

Gibson, K. D., Segen, B. J. & Audhya, T. K. (1977) *Biochem. J.* **162**, 217–233

Handley, C. J. & Lowther, D. A. (1976) *Biochim. Biophys. Acta* **444**, 69–74

Handley, C. J. & Lowther, D. A. (1977) *Biochim. Biophys. Acta* **500**, 132–139

Handley, C. J., Bateman, J. F., Oakes, B. W. & Lowther, D. A. (1975) *Biochim. Biophys. Acta* **386**, 444–450

Hardingham, T. E. & Muir, H. (1970) *FEBS Lett.* **9**, 145–148

Hardingham, T. E., Fitton-Jackson, S. & Muir, H. (1972) *Biochem. J.* **129**, 101–112

Hopwood, J. J. & Robinson. H. C. (1973) *Biochem. J.* **135**, 631–637

Lane, J. M. & Brighton, C. T. (1974) *Arth. Rheum.* **17**, 235–243

Lane, J. M., Brighton, C. T. & Menkowitz, B. J. (1977) *J. Rheumatol.* **4**, 334–342

Lemperg, R., Larsson, S. & Jones, I. (1977) *Biochim. Biophys. Acta* **496**, 420–430

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Lowther, D. A., Sandy, J. D., Santer, V. B. & Brown, H. L. G. (1978) *Arth. Rheum.* **21**, 675–680

Mankin, H. J. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1478–1480

Mankin, H. J. & Lippiello, L. (1970) *J. Bone Surg. Am.* **52**, 424–434

Maroudas, A. & Evans, H. (1974) *Biochim. Biophys. Acta* **338**, 265–279

McDevitt, C. A., Muir, H. & Pond, M. J. (1973) *Biochem. Soc. Trans.* **1**, 287

Millroy, S. J. & Poole, A. R. (1974) *Ann. Rheum. Dis.* **33**, 500–508

Mitrovic, D., Gruson, M., Demignon, J. & Cohen-Solal, L. (1978) *Cell Tissue Res.* **186**, 149–159

Nevo, Z., Silbergeld, A. K. & Mamet, R. (1978) *Biochim. Biophys. Acta* **541**, 76–83

Pearson, J. P. & Mason, R. M. (1977) *Biochim. Biophys. Acta* **498**, 176–188

Robinson, H. C. & Dorfman, A. (1969) *J. Biol. Chem.* **244**, 348–352

Robinson, H. C. & Hopwood, J. J. (1973) *Biochem. J.* **133**, 457–470

Robinson, H. C., Brett, M. J., Tralaggan, P. J., Lowther, D. A. & Okayama, M. (1975) *Biochem. J.* **148**, 25–34

Rosenberg, L., Wolfenstein-Todel, C., Margolis, R., Pal, S. & Strider, W. (1976) *J. Biol. Chem.* **251**, 6439–6444

Royce, P. M. & Lowther, D. A. (1979) *Connect. Tissue Res.* **6**, 215–221

Sandy, J. D. (1979) *Biochem. J.* **177**, 569–574

Sandy, J. D., Brown, H. L. G. & Lowther, D. A. (1977) *Proc. Aust. Biochem. Soc.* **10**, 46

Sandy, J. D., Brown, H. L. G. & Lowther, D. A. (1978) *Biochim. Biophys. Acta* **543**, 536–544

Schwartz, N. B. (1974) *FEBS Lett.* **49**, 342–345

Schwartz, N. B. (1976) *J. Biol. Chem.* **251**, 3346–3351

Schwartz, N. B. & Rodén, L. (1974) *Carbohydr. Res.* **37**, 167–180

Simunek, Z. & Muir, H. (1972) *Biochem. J.* **126**, 515–523

Stoolmiller, A. C., Horwitz, A. L. & Dorfman, A. (1972) *J. Biol. Chem.* **247**, 3525–3532

Wiebkin, O. & Muir, H. (1973) *FEBS Lett.* **37**, 42–46