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Chondrocyte-mediated depletion of articular cartilage proteoglycans *in vitro*

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The degradation of proteoglycan was examined in cultured slices of pig articular cartilage. Pig leucocyte catabolin (10ng/ml) was used to stimulate the chondrocytes and induce a 4-fold increase in the rate of proteoglycan loss from the matrix for 4 days. Material in the medium of both control and depleted cultures was mostly a degradation product of the aggregating proteoglycan. It was recovered as a very large molecule slightly smaller than the monomers extracted with 4M-guanidinium chloride and lacked a functional hyaluronate binding region. The size and charge were consistent with a very limited cleavage or conformational change of the core protein near the hyaluronate binding region releasing the *C*-terminal portion of the molecule intact from the aggregate. The 'clipped' monomer diffuses very rapidly through the matrix into the medium. The amount of proteoglycan extracted with 4M-guanidinium chloride decreased during culture from both the controls and depleted cartilage, and the average size of the molecules initially remained the same. However, the proportion of molecules with a smaller average size increased with time and was predominant in explants that had lost more than 70% of their proteoglycan. All of this material was able to form aggregates when mixed with hyaluronate, and glycosaminoglycans were the same size and charge as normal, indicating either that the core protein had been cleaved in many places or that larger molecules were preferentially released. A large proportion of the easily extracted and non-extractable proteoglycan remained in the partially depleted cartilage and the molecules were the same size and charge as those found in the controls. There was no evidence of detectable glycosidase activity and only very limited sulphatase activity. A similar rate of breakdown and final distribution pattern was found for newly synthesized proteoglycan. Increased amounts of latent neutral metalloproteinases and acid proteinase activities were present in the medium of depleted cartilage. These were not thought to be involved in the breakdown of proteoglycan. Increased release of proteoglycan ceased within 24h of removal of the catabolin, indicating that the effect was reversible and persisted only while the stimulus was present.

Collagen and proteoglycan provide articular cartilage with unique physical properties of tensile strength and reversible compressibility that enable it to withstand mechanical stress and protect the underlying bone (Kempson, 1975). It is the loss of these two structural components that leads to the inevitable crippling associated with arthritis.

Cartilage can be grown as healthy, viable explants in organ culture for several weeks (Tyler *et al.*, 1982). During this period a fairly stable equilibrium for the matrix is established. A basal level of proteoglycan is broken down, causing a stimulation of glycosaminoglycan synthesis (Sandy *et al.*, 1980). The amount released varies depending on the species, age of the explants, and serum content of the medium. Removal of the matrix proteoglycan with enzymes such as hyaluronidase and trypsin leads to a surge of new synthesis and replacement of some of the lost material (Hardingham *et al.*, 1972; Millroy & Poole, 1974). Very little is known about the mechanism chondrocytes use to regulate and maintain this balance. It can be shifted towards net degradation in the absence of extrinsic enzymes by agents which stimulate chondrocytes to induce resorption, such as retinol (Fell

Abbreviation used: APMA, 4-aminophenylmercuric acetate.

et al., 1956; Lucy *et al.*, 1961; Jubb & Fell, 1980), bacterial lipopolysaccharides (Jasin, 1984; Morales *et al.*, 1984), and products of cultured synovial (Fell & Jubb, 1977; Dingle *et al.*, 1979; Steinberg *et al.*, 1979; Pilsworth & Saklatvala, 1983; Klamfeldt *et al.*, 1982) and mononuclear cells (Saklatvala & Sarsfield, 1982; Jasin & Dingle, 1981).

One of these factors, pig leucocyte catabolin, has been purified from the medium of mononuclear cells stimulated with lectins. It is a protein with an M_r of 210000 and an isoelectric point of 4.9, and does not have independent proteolytic activity (Saklatvala *et al.*, 1983). When cultured with cartilage explants catabolin causes a dose-dependent (10^{-9}–10^{-11} M) increase in the rate of proteoglycan depletion. It was of interest to know why proteoglycan had been released from the matrix, whether the mechanism during stimulation was the same as in the controls and whether there was any change in the proteoglycan remaining in the partially depleted explants.

In the present paper I describe how fractionated material containing catabolin was used to initiate chondrocyte-mediated depletion of cultured articular cartilage. Proteoglycans released into the medium and those remaining in the explant were isolated and characterized. An understanding of how chondrocytes respond to molecules such as catabolin is essential before one can assess whether this type of activity is important in diseases characterized by progressive erosion of cartilage and bone.

Materials and methods

Materials

Iscove's modified Eagle's medium and supplements were from Gibco. 1,9-Dimethyl Methylene Blue was from Serva Feinbiochemica, Heidelberg, Germany. Keratan sulphate, chondroitin sulphate and chondroitinase ABC were from Miles Laboratories, Stoke Poges, Slough, Berks., U.K. [^{35}S]-Sulphate was from The Radiochemical Centre, Amersham, Bucks., U.K. Sepharose CL-2B and Sephacryl S-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Pepstatin was from Peptide Institute, Osaka, Japan. Human cathepsin D was a gift from Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge. All other reagents were of analytical reagent grade.

Tissue culture

Forefeet from pigs aged about 25 weeks were supplied by a local slaughterhouse. Cartilage explants were made by cutting a long, thin strip (approx. 12mm × 2mm × 0.3mm) from the condylar ridge, care being taken to exclude the underlying marrow. The strip was cut transversely into two equivalent pieces (7–11mg wet wt.). One half of the pair received the experimental treatment and the other served as a control. Six to eight halves were usually placed in Petri dishes (25mm) which contained 1ml of Iscove's medium supplemented with bovine serum albumin (60μg), human transferrin (5μg), soya bean lipid (15μg), ascorbic acid (100μg), streptomycin (100μg), and penicillin (150 units).

Cultures were incubated at 37°C in an atmosphere of CO_2/air (1:19) and the medium, changed every 8–16h, was collected separately for analysis. Explants were cultured for 48h to stabilize prior to treatment with catabolin (day 1). Medium used for radiolabelling experiments contained 5% of normal levels of sulphate.

Fractionation of pig mononuclear cell medium

The fractions used to stimulate cartilage depletion contained approx. 10ng of catabolin/ml, and were generously given by Dr. J. Saklatvala, Strangeways Research Laboratory, Cambridge. They were isolated from the conditioned medium of cultured pig mononuclear cells stimulated with lectins. Protein in the culture medium was concentrated by ultrafiltration, chromatographed on Ultrogel AcA54 and chromatofocused on a prepacked MonoP HR5/20 column eluted with Polybuffer 74. Active fractions (M_r 21000, pI4.8–5.0) were then chromatographed on hydroxyapatite and dialysed against culture medium using acetylated Visking tubing which had previously been immersed in 25% acetic anhydride in pyridine (Saklatvala *et al.*, 1983).

Labelling and extraction of glycosaminoglycans

Cartilage slices were prelabelled by incubation with [^{35}S]sulphate (20μCi/ml) at 37°C for 16h in low-sulphate medium. The slices were washed and incubated twice in normal medium (16h, 37°C), and cut into two. The explant halves were cultured in the presence or absence of 10ng of catabolin/ml at 37°C for 96h. Cryostat sections (30μm) of the control and depleted cartilage halves were sequentially extracted twice with 0.5M-guanidinium chloride in 0.05M-sodium acetate buffer, pH5.8 (low salt), then twice with 4M-guanidinium chloride in 0.05M-sodium acetate buffer, pH5.8 (high salt) at 3°C for 24h. The residues were washed with water and solubilized with papain (300μg/ml) in 0.05M-phosphate buffer, pH6.5, containing 2mM-*N*-acetylcysteine and 2mM-EDTA at 65°C for 1h. The following inhibitors were present in all the extraction and dialysis procedures: pepstatin (1μg/ml), 1,10-phenanthroline (1mM), iodoacetic acid (1mM) phenylmethanesulphonyl fluoride (1mM) and toluene (0.03%). The medium samples,

low-salt extract, high-salt extract and digested residues were dialysed against water at 4°C, lyophilized and stored at −20°C.

Gel chromatography

The size distribution of proteoglycans in the medium and extracts was determined by gel chromatography on columns (100cm × 0.66cm) of Sepharose CL-2B (Heinegard, 1972) eluted with 0.5M-sodium acetate buffer, pH7.0, at 22°C and 4ml/h in the presence and absence of 4M-guanidinium chloride. Samples to be tested for the ability to aggregate were first mixed with hyaluronic acid (2% of the sample uronic acid) at 4°C for 4h.

The size distribution of glycosaminoglycan chains was determined on a column (100cm × 0.66cm) of Sephacryl S-200 (Wasteson, 1971) after treating samples with papain or alkali [1M-$NaBH_4$ in 0.05M-NaOH at 45°C for 48h (Carlson, 1968)]. Proteoglycan aggregates and [^{35}S]sulphate were used as markers of the void (V_0) and total (V_t) volumes of the columns. Proteoglycan aggregate was prepared from bovine nasal cartilage by CsCl density centrifugation (Hascall & Sajdera, 1969).

The effluent fractions were analysed for radioactivity and glycosaminoglycans.

Electrophoresis of glycosaminoglycans on cellulose acetate

The distribution of sulphate residues in purified chondroitin sulphate chains was determined as described by Wasteson & Lindahl (1971). Proteoglycan samples eluted from Sepharose 2B columns were hydrolysed with alkali, neutralized and applied to columns of Sephacryl S-200. The eluate was divided into four equal fractions which were dialysed against water and lyophilized. Duplicate samples were applied to pre-soaked cellulose acetate strips (5cm × 20cm; Celagram II; Shandon Southern, Runcorn, Cheshire, U.K.). Samples (1mg/ml) of hyaluronic acid, chondroitin sulphate, keratan sulphate and heparin were run as standards. Electrophoresis was performed in 0.1M-HCl at 7.5V/cm for 2h. One sample strip was cut into 0.2cm sections for determination of radioactivity. The other was stained with the standards in a solution of Alcian Blue (0.02%) in 0.05M-$MgCl_2$/0.05M-sodium acetate buffer, pH5.8, for 30min at room temperature, then destained in the same buffer without Alcian Blue.

Proteinase assays

^{35}S-labelled proteoglycan aggregate entrapped in polyacrylamide beads (Dingle *et al.*, 1977) was used as a substrate in an assay volume of 250μl. Conditioned medium (up to 100μl) was incubated at 37°C for 18h on a rotary stirrer to ensure good mixing with the beads. Assays were performed with 10mM-$CaCl_2$ in 50mM-Tris/HCl buffer, pH7.4, or with 10mM-sodium acetate buffer, pH4.5. APMA (0.3M), 1,10-phenanthroline (2mM), pepstatin (1μg/ml) and di-isopropylphosphofluoridate (5mM) were included in some of the assays. The reaction was terminated by the addition of an inhibitor cocktail and the beads were precipitated by centrifugation for 5min.

The percentage release of radiolabel was calculated after papain digestion of the beads and compared with a standard curve of log(cathepsin D concentration). The human cathepsin D had a specific activity of 750 units/mg (1 unit = ΔA_{280} 1.0/h; Barrett, 1970).

Analytical procedures

Glycosaminoglycan was estimated from the amount of polyanionic material reacting with 1,9-dimethyl Methylene Blue (Farndale *et al.*, 1982) and as uronic acid by the method of Bitter & Muir (1962). Shark chondroitin sulphate and glucuronolactone were used as a standard. Glucose was estimated from the formation of NADPH after conversion to glucose 6-phosphate by hexokinase in the presence of ATP (Sigma kit 15-u.v.).

Collagen was estimated as hydroxyproline in neutralized acid hydrolysates of the medium or tissue (6M-HCl, 105°C, 20h) as described by Tougaard (1973). DNA was measured in papain digests of the tissue by the method of Royce & Lowther (1979).

Radioactive samples (up to 250μl) were mixed with 2.5ml of scintillant solution (50% Picofluor in toluene) and counted on a Packard Tricarb-300 scintillation counter. The counts were corrected to d.p.m. by using an external standard.

Results

A series of paired ^{35}S-labelled explants was prepared from slices of pig articular cartilage, half being grown in control medium and the other in medium containing catabolin as described under 'Materials and methods'. The medium, changed every 8–16h, was mixed with proteinase inhibitors and kept separately for analysis. A constant level of glycosaminoglycan was released into the medium of control cultures during the 3 days (Fig. 1*b*). A catabolin concentration (10ng/ml) was chosen which increased the rate of glycosaminoglycan loss by about 4-fold. This effect is dose-dependent; a higher concentration will increase the rate still further and a lower dose will reduce it. The time taken to release 50% of the proteoglycan into the medium varied by ±1 day in different batches of cartilage. The results were therefore usually com-

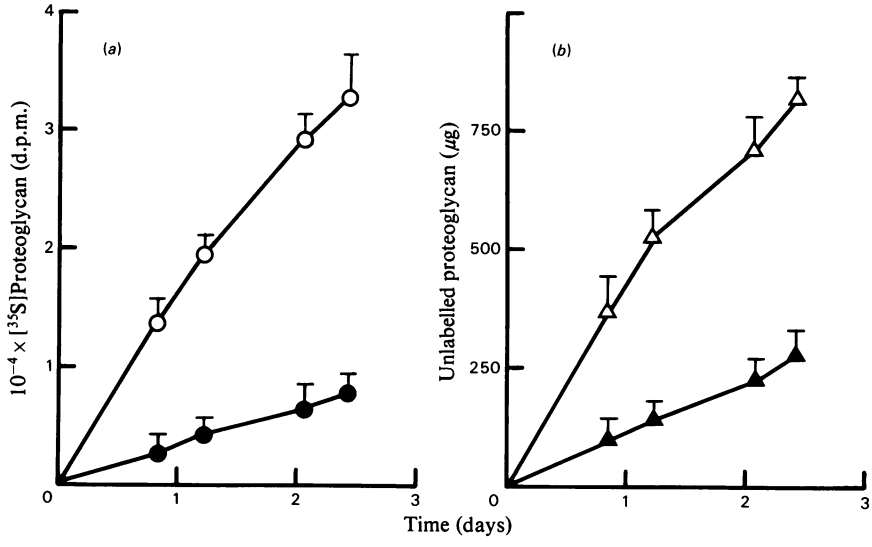


Fig. 1. *Cumulative release of newly synthesized and unlabelled glycosaminoglycans into the medium of control and depleted cartilage cultures*

Culture medium from control (●, ▲) and depleted (○, △) prelabelled cartilage was assayed for (*a*) cetyl pyridinium chloride-insoluble ^{35}S ([^{35}S]glycosaminoglycan) and (*b*) for dimethyl Methylene Blue reactive material (glycosaminoglycan mass). The values represent the average of three experiments each using six pairs of explants. Bars are S.E.M.

Table 1. *Distribution of newly synthesized and unlabelled glycosaminoglycans in the medium and cartilage of control and depleted cultures*

Sections (30 μm) of the control and depleted cartilage were sequentially extracted twice with low salt (0.05M-guanidinium chloride), then high salt (4M-guanidinium chloride) in 0.05M-sodium acetate, pH 5.8, at 30°C for 24h. The residue was solubilized with papain. ^{35}S-labelled and unlabelled glycosaminoglycans in each extract and the medium are shown as an absolute amount per culture (±S.E.M.) and as a percentage of the total. The values represent the average of three experiments each using six pairs of explants.

	Unlabelled glycosaminoglycans (μg/culture)		$10^{-4} \times$ [^{35}S]Glycosaminoglycans (d.p.m./culture)	
Source	Control	Depleted	Control	Depleted
Medium	259±27 (16.1)	780±31 (51)	76±14 (13)	327±39 (54)
Low salt extract	254±77 (15.9)	214±48 (14)	99±21 (17)	85±10 (14)
High salt extract	928±61 (58)	428±66 (28)	372±34 (64)	163±21 (27)
Residue	160±59 (10)	107±29 (7)	35±4 (6)	30±3 (5)

pared by percentage depletion rather than days in culture.

Distribution of glycosaminoglycan in the media and extracts

The control and depleted cartilage was sliced into 30μm sections which were sequentially extracted with 0.5M-guanidinium chloride in 0.05M-acetate, pH 5.8, for 24h (×2), then with 4M-guanidinium chloride in 0.05M-acetate, pH 5.8, for 24h (×2). The residue was solubilized with papain. The distribution of unlabelled glycosaminoglycan as a percentage of the total is shown in Table 1. The proportion of proteoglycan in the high salt extract of depleted cartilage was reduced compared with

the controls, whereas the amount of material extracted with low salt buffer or solubilized from the residue of depleted cartilage was very similar to that of the control cartilage.

The newly synthesized glycosaminoglycans show a similar distribution (Fig. 1*a*, Table 1), the rate of release from the stimulated cartilage being the same as for the unlabelled material.

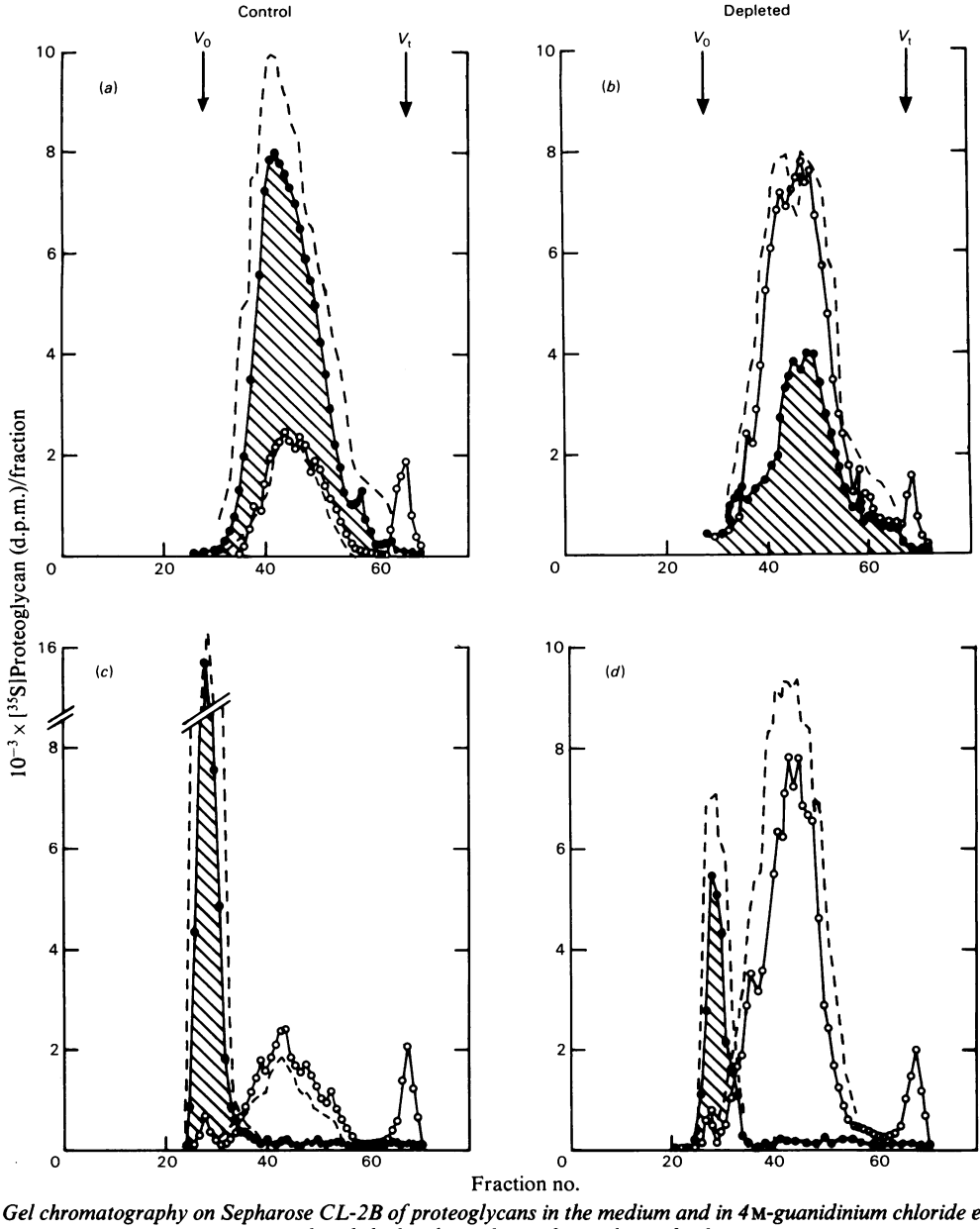


Fig. 2. *Gel chromatography on Sepharose CL-2B of proteoglycans in the medium and in 4M-guanidinium chloride extracts of control and depleted cartilage after 4 days of culture*

Samples of proteoglycan from conditioned medium (white areas) and 4M-guanidinium chloride extracts (shaded areas) were eluted from a column of Sepharose CL-2B in the presence of 4M-guanidinium chloride; (*a*) control, (*b*) depleted. Duplicate samples were dialysed, mixed with hyaluronate for 4h at 4°C and eluted from a column of Sepharose CL-2B; (*c*) control, (*d*) depleted. All the columns (100cm × 0.66cm) were eluted with 0.5M-sodium acetate, pH6.8, at the rate of 4ml/h at room temperature. Fractions were analysed for dimethyl Methylene Blue reactive material (---) and radioactivity (●, ○). V_0 indicates the void volume and V_t the total bed volume of the columns.

Gel chromatography of the proteoglycan released into the medium and extracted with high salt

The average molecular size and ability to aggregate of proteoglycan released into the medium and extracted with high salt is shown in Fig. 2. The dotted lines represent unlabelled (μg) and the solid line newly synthesized (d.p.m.) proteoglycan.

Material released into the medium of control cultures was eluted from Sepharose 2B in the presence of 4M-guanidinium chloride. It was recovered as a very large molecule (Fig. 2*a*, white area) slightly smaller than the monomers in the high salt extract (Fig. 2*a*, shaded area). If mixed with hyaluronate and eluted under associative conditions only 3.7% of the medium sample, but most of the high salt extract, was able to aggregate (Fig. 2*c*). Proteoglycan in the medium of depleted cultures was the same large size as the controls (Fig. 2*b*, white area), and only 2.1% aggregated in the presence of hyaluronate (Fig. 2*d*). Proteoglycan in high salt extracts of depleted cartilage included more molecules with a smaller average size (Fig. 2*b*, shaded area) than those from the control. All of this material retained a functional hyaluronate-binding region and was eluted in the void volume when mixed with hyaluronate (Fig. 2*d*).

A small amount of radiolabelled material was recovered as a very low M_r peak. This eluted as free sulphate from a Sephadex G-25 column (results not shown) and may be the product of limited sulphatase activity. It represented less than 5% of total radiolabelled material and was not increased in the depleted cultures, indicating that newly synthesized material was not preferentially degraded.

To analyse the extent to which molecules remaining in the matrix were degraded, five groups of paired ^{35}S-prelabelled cartilage halves were stimulated to resorb their proteoglycan for 0, 1, 2.5, 3.5 and 7 days. The explants and their controls were then sliced and extracted as before. The distribution of radiolabelled molecules at different stages of depletion was measured and is shown in Fig. 3 as the mean of three experiments.

For the cartilage stimulated to resorb (open circles), increased release of proteoglycan into the

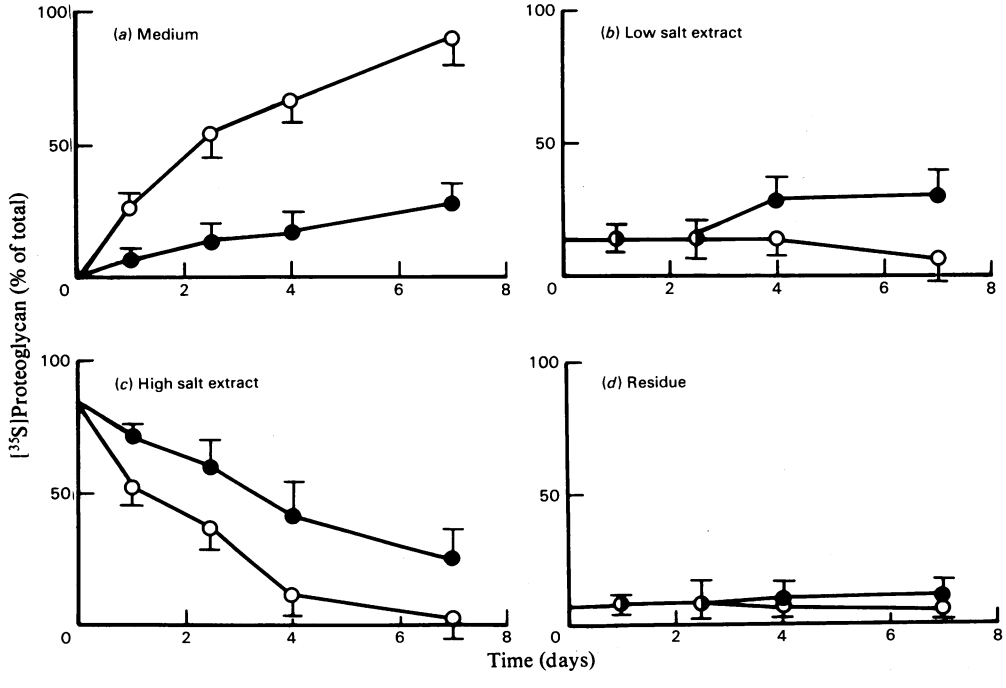


Fig. 3. *Distribution of proteoglycans in the medium and extracts of control and depleted cartilage at different times*
Four groups of six cartilage pairs were cultured in the presence (○) or absence (●) of catabolin for 1, 2.5, 3.5 or 7 days. The amount of [^{35}S]glycosaminoglycans in (*a*) medium, (*b*) high salt extract, (c) low salt extract and (*d*) residue was measured and is shown as a percentage of the total for each time point. The values represent the average of three experiments. Bars indicate the range.

medium (Fig. 3*a*) was accompanied by a parallel decrease in the amount of large aggregating proteoglycan extracted with high salt (Fig. 3*b*). The proportion of easily extracted material in the low salt extracts remained constant (Fig. 3*d*) at 15–17% for the first three time points and was reduced to 5% in the severely depleted cartilate.

In the controls (solid circles), the rate of proteoglycan loss from the high salt extracts (Fig. 3*b*) was much more than that predicted from the rate of release into the medium (Fig. 3*a*). This was due to an increase in the proportion of easily extracted material from 16% to 30% (Fig. 3*c*). The amount of inextractable proteoglycan solubilized from the residue with papain remained fairly constant in both cultures at 4–10% (Fig. 3*d*).

The elution profile from Sepharose 2B of samples from the high salt extracts show that the average molecular size of the controls remains the same (Figs. 4*a*–4*d*). The average size of molecules from the stimulated cartilage was initially the same as the controls (Fig. 4*a*). However the proportion of small molecules increased with time (Fig. 4*b*) and they were predominant in material extracted from severely depleted cartilage (Figs. 4*c* and 4*d*).

Size and degree of sulphation of chondroitin sulphate isolated from the proteoglycans in the high salt extract and medium

Samples eluting within the peaks shown in Figs. 2(*a*) and 2(*b*) were pooled, dialysed, digested with papain and lyophilized. Aliquots were then hydrolysed with alkali in the presence of $NaBH_4$. The elution profiles from Sephacryl S-200 of the glycosaminoglycan chains are shown in Fig. 5. The average molecular size of the chains from the high salt extracts and medium of the depleted cartilage was the same as the controls. Chondroitin sulphate chains isolated from the small proteoglycans eluting as shown in Figs. 4(*b*)–4(*d*) were also the same size as the controls (results not shown).

Samples from the alkali-hydrolysed peaks were pooled as indicated by the bars, dialysed, lyophilized and analysed by electrophoresis in HCl. This detects differences in the distribution of sulphate which might result from the action of hyaluronidase or sulphatase (Wasteson & Lindahl, 1971). Glycosaminoglycans from a range of sizes across the peaks from the high salt extracts migrated with the same mobility (Figs. 6*b* and 6*d*) corresponding to the stained sample. This suggests that there is no significant change in the degree of sulphation of the chains. Chains prepared from the medium samples, in addition to a major peak corresponding to normal chondroitin sulphate, contained a less mobile species (Figs. 6*a* and 6*c*). This was seen in the medium from both control and depleted cultures.

Gel chromatography of proteoglycans in the low salt extract and glycosaminoglycans solubilized from the residue

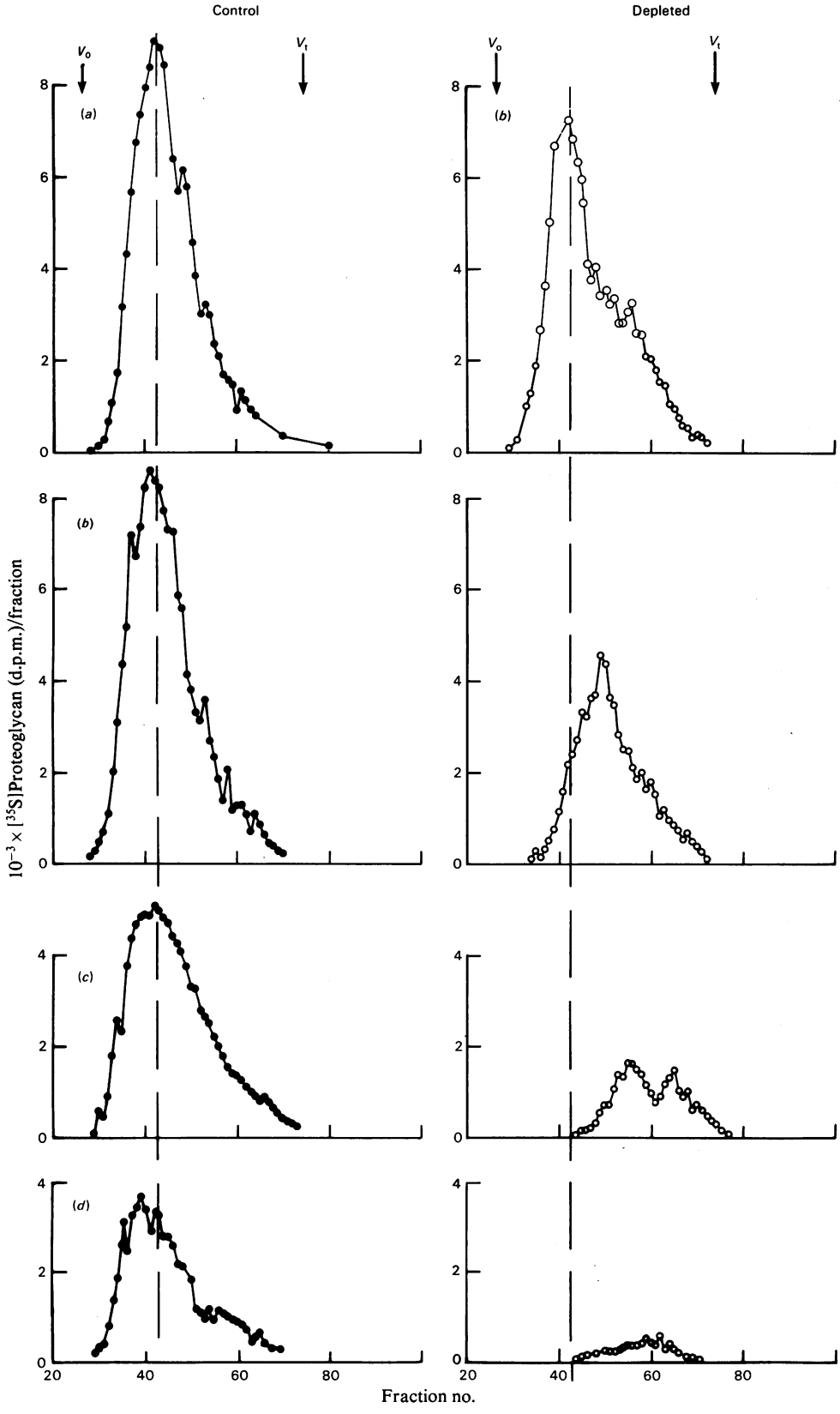
The elution profiles from Sepharose 2B of the material extracted at low ionic strength is shown in Fig. 7(*b*). Samples from the depleted cartilage are the same size as the controls, both being smaller than the average monomers isolated in the preparations from the high salt extracts. If the samples were first mixed with hyaluronic acid (2% based on uronic acid content) 20.4% of the control (Fig. 7*a*) and 25.2% of the depleted material was eluted in the void volume. The chondroitin sulphate chains from papain digests of both the control and depleted extract (Fig. 7*c*) were the same size, slightly longer than those from the high salt extract (Fig. 5). Glycosaminoglycans solubilized from the residue of depleted cartilage with papain were the same size as the controls (Fig. 7*d*).

Analysis of media and cartilage

In a parallel set of experiments, the collagen and DNA content and glucose utilization of control and depleted cultures were determined (Table 2). Less than 2% of the total hydroxyproline was found in the medium, indicating that there is no loss of collagen from the matrix at this time. There was also no marked difference in the cellularity of the depleted cartilage (μg of DNA). The metabolic rate of control and depleted cultures as measured by loss of glucose from the medium was not significantly different.

Table 2. *Analysis of cartilage and medium*
Six paired cartilage halves were cultured for 4 days in the presence (depleted) and absence (control) of catabolin. The medium and papain digests of the cartilage were analysed for DNA, collagen, glycosaminoglycan and glucose. The data represents the mean of twelve groups ± S.E.M. Values in parentheses are 100% × [μg (cartilage)/μg (cartilage + medium)].

Constituent	Amount (μg/culture) Control	Depleted
Proteoglycan (dimethyl Methylene Blue)	1342 ± 176 (83%)	750 ± 105 (49%)
Proteoglycan (uronic acid)	773 ± 89 (89%)	427 ± 67 (54%)
Collagen (hydroxyproline)	954 ± 74 (98.6%)	908 ± 109 (98.8%)
DNA	18 ± 2.2	19.4 ± 0.4
Glucose (loss from medium in 4 days)	10.7%	11.8%


Control
Depleted
V_o
V_t
(a)
(b)
(b)
(c)
(d)
$10^{-3} \times$ [^{35}S]Proteoglycan (d.p.m.)/fraction
Fraction no.
0
2
4
6
8
20
40
60
80

Proteinase in the medium

Medium samples were analysed for the presence of acid (pH4.5) and neutral (pH7.4) proteinases which could degrade proteoglycan. Increased levels of both types of activity were found in depleted cultures compared with the controls (Figs. 8*a* and 8*b*). The neutral activity was only evident if AMPA (0.7mM) was included in the assays and was reduced by 1,10-phenanthroline. The acidic activity was reduced by pepstatin (1 μg/ml) and by prolonged storage.

Conditioned medium from depleted cultures containing the large 'clipped' monomers as shown in Fig. 2(b) was incubated at pH7.4 in the presence of APMA and at pH4.5. A profile of the digestion products is shown in Figs. 8(*c*) and 8(*d*). For comparison the size of the starting material (shaded area) and products obtained by digestion of the same sample with trypsin (dotted line) are also shown. A range of smaller molecules were generated at both neutral (Fig. 8*c*) and acid (Fig. 8*d*) pH.

The proteoglycan in the medium of cartilage cultures is therefore not resistant to proteolysis, but was not further degraded by enzymes in the untreated medium of depleted cultures.

Release of proteoglycan during recovery

Paired explants were stimulated to resorb their proteoglycans for 1, 2, 3 or 4 days. The cartilage was then washed and allowed to recover in control

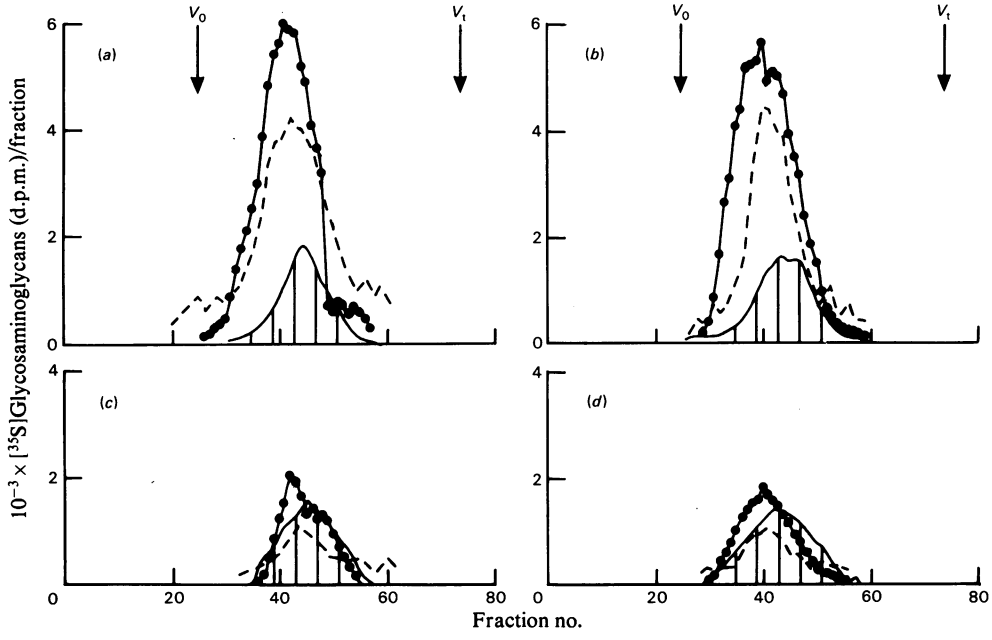


Fig. 5. *Comparison of the average molecular size of glycosaminoglycans isolated from proteoglycan in the high salt extracts and the medium*

Samples of proteoglycan from the medium and high salt extracts of control and depleted cartilage (Figs. 3*a* and 3*b*) were dialysed, digested with papain or hydrolysed with alkali in the presence of $NaBH_4$. Fractions were eluted from a column of Sephacryl S-200. (*a*) Medium (depleted), (*b*) extract (control), (*c*) medium (control), (*d*) extract (depleted). Papain digest: ●, [^{35}S]glycosaminoglycans: ---, unlabelled glycosaminoglycan. Alkali hydrolysis: ——, [^{35}S]glycosaminoglycan.

Fig. 4. *Comparison of the average molecular size of proteoglycans extracted with 4M-guanidinium chloride from control and depleted cartilage at different times.*

Samples of proteoglycan in the high salt extracts of cartilage (Fig. 3*b*) after (*a*) 1 (*b*) 2.5, (*c*) 4 and (*d*) 7 days of culture were eluted from a column of Sepharose CL-2B as described for Fig. 2. ●, Control; ○, depleted.

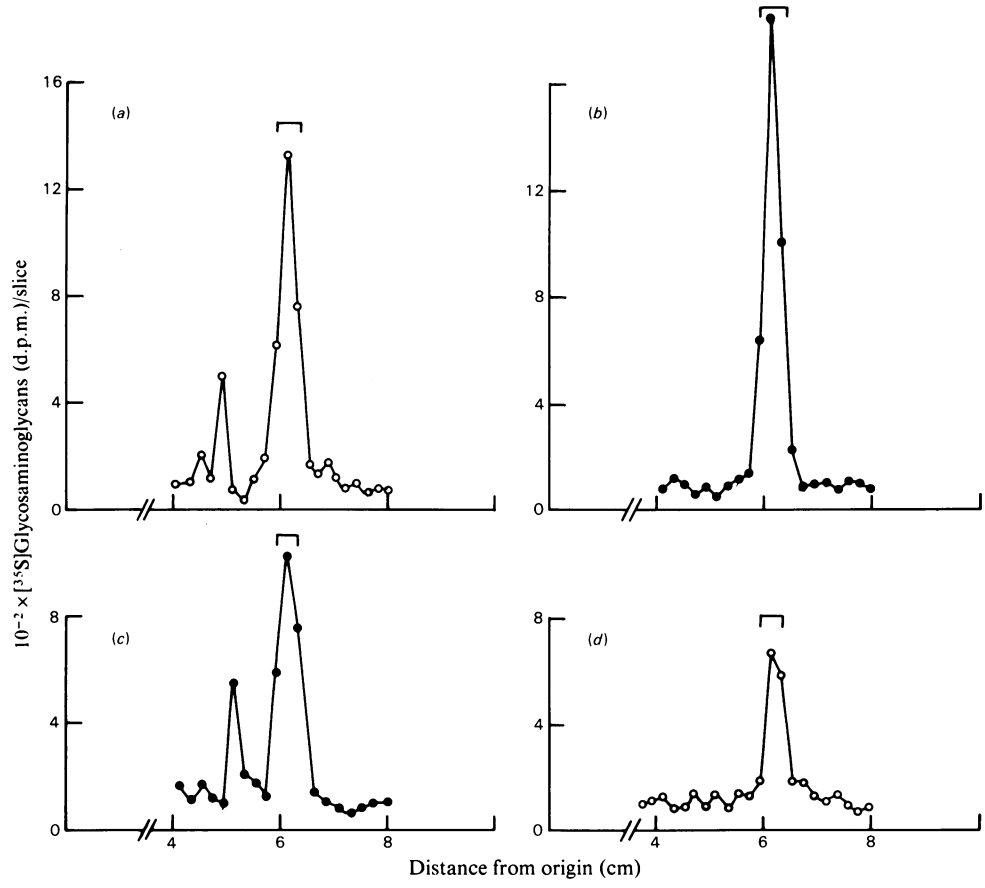


Fig. 6. *Electrophoresis of the glycosaminoglycan chains*

Each peak of the alkali-hydrolysed sample from Fig. 5 was divided into four size fractions as indicated by the vertical lines. The fractions were pooled, dialysed and lyophilized. Duplicate samples were subjected to electrophoresis on strips of cellulose acetate in HCl. One strip was stained and the other was cut into 0.2 cm slices and analysed for radioactivity. The position of the stained sample is denoted by a bracket (⌐).

medium. The rate of proteoglycan loss was reduced to that of the control by 24h at each time tested (Fig. 9; day 2 only shown). The catabolin effect is therefore reversible and persists only if the factor is continually present.

Discussion

The experiments reported here show that the glycosaminoglycan released into the medium during chondrocyte-mediated resorption of the cartilage matrix consisted of very large molecules, slightly smaller than monomers, which failed to reaggregate in the presence of hyaluronate. Similar products were generated from both control cartilage and that stimulated with catabolin. The same large product has been found in the culture medium of rabbit articular cartilage (Handley & Lowther, 1977; Sandy *et al.*, 1978) and chick limb bones treated with vitamin A (Morrison, 1970). Radiolabelled proteoglycan was released from stimulated cartilage as the same sized molecule and with a similar increase in rate as the unlabelled proteoglycan. A small amount (<5%) of very low M_r material was recovered in the medium as sulphate, but no more than in the controls, indicating that the recently synthesized proteoglycan was not preferentially degraded.

A time course of the distribution pattern, and the size of the monomers and chondroitin sulphate chains, suggests that most of the proteoglycan in the medium is a degradation product of the large aggregating proteoglycan. The results are consistent either with a very limited cleavage of the core protein at a site near the hyaluronate binding region, releasing the rest of the molecule intact

from the aggregate, or with deaggregation then inactivation of the binding region. A large accumulation of these clipped monomers was never found within the explants, indicating that they have rapidly diffused through the matrix into the medium. It is not known whether deaggregation occurs prior to cleavage, and the fate of the hyaluronate binding region, which is mostly devoid of glycosaminoglycans and the link protein, is at present unknown.

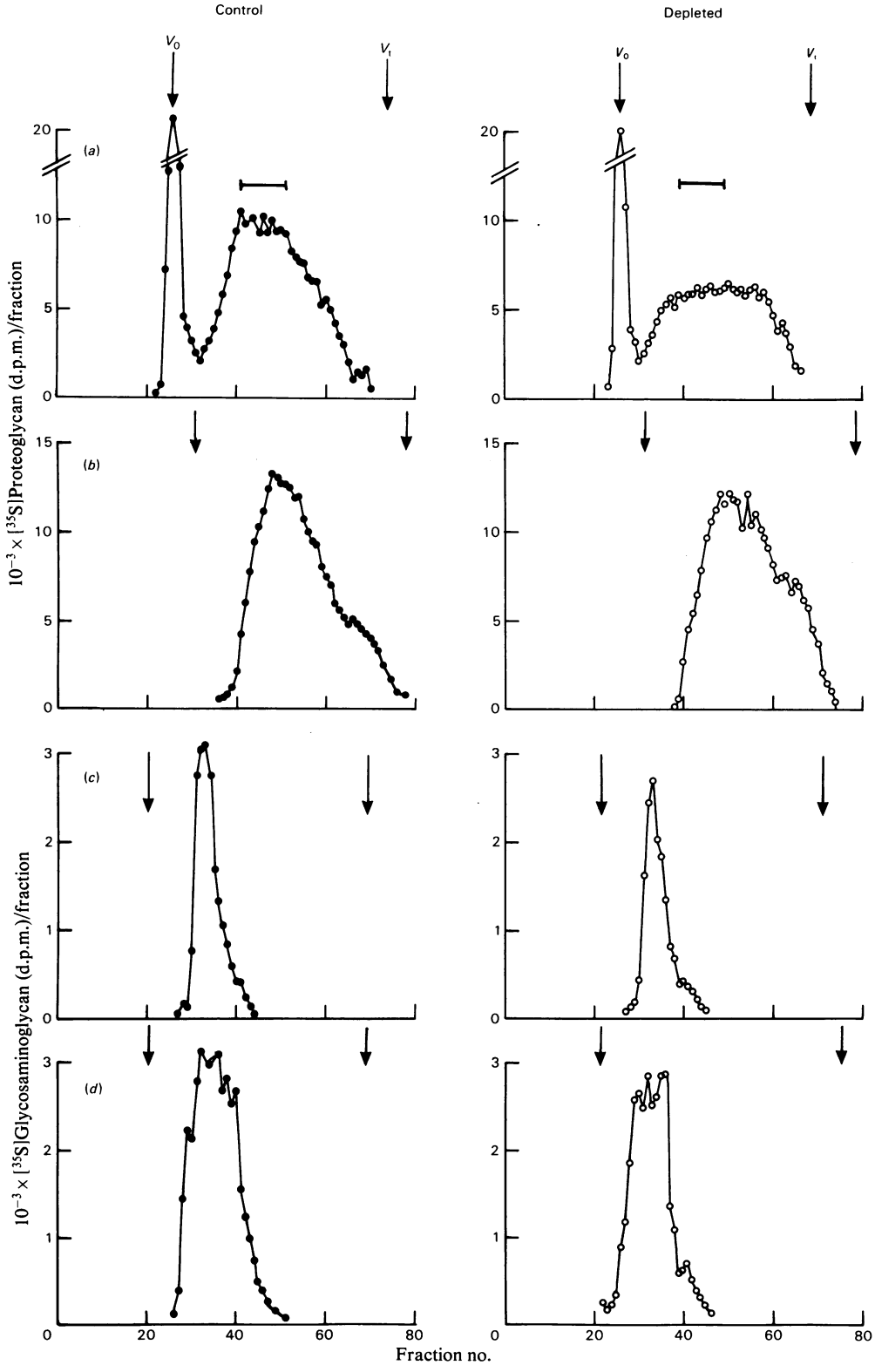
Release of glycosaminoglycan into the medium was accompanied by a decrease in the amount of the aggregating proteoglycan extracted with high salt. At high rates of depletion a large proportion of the monomers left in this fraction were of a much smaller size. All of this material retained a functional binding region and was able to reaggregate in the presence of hyaluronate. Chondroitin sulphate chains isolated from the smaller proteoglycan were the same as normal, and the distribution of sulphate on size-matched samples was the same as in the controls as judged by electrophoresis in HCl. There was no evidence of glycosidase activity. This suggests either that the core protein of molecules remaining in the depleted cartilage has been cleaved in more than one place, or that larger molecules are preferentially released from the aggregates. Intact polysaccharide chains were also found after degradation of rat costal cartilage *in vivo* and *in vitro* (Wasteson *et al.*, 1972).

The proportion and size of the small non-aggregating proteoglycans remaining in the partially depleted explants is typical of proteoglycan extracted with low ionic strength buffers from several types of cartilage (Tsiganos & Muir, 1969; Hardingham & Muir, 1976; Simunek & Muir, 1972; Heinegard, 1977). The average molecular size of this material is very similar to that recovered from the medium and it is not clear why such molecules remain within the cartilage. Presumably they are located in a region which prevents rapid diffusion through the matrix or are anchored by association with collagen.

The core protein of cartilage proteoglycan contains several sites between groups of glycosaminoglycan chains which are very susceptible to cleavage by the majority of proteinases. Further cleavage within these groups to liberate smaller glycosaminoglycan clusters then depends on the type of proteinase. Two major products are found after prolonged digestion. One, a peptide bearing single keratan sulphate or chondroitin sulphate chains, results from the action of enzymes with a broad specificity such as papain and cathepsin B. In the other, two to six chondroitin sulphate chains remain attached to the peptide, characteristic of digestion with trypsin, cathepsin D, neutral metalloproteinase and most other proteinases (Roughley, 1977; Roughley & Barrett, 1977; Morrison *et al.*, 1973; Galloway *et al.*, 1983; Heinegard & Hascall, 1974). Increased levels of at least two types of proteinase capable of degrading proteoglycan were present in the culture medium of depleted explants. The activity measured at acid pH was inhibited by pepstatin and is probably cathepsin D. The neutral activity, a metalloenzyme, was only seen after treatment of the proenzyme with APMA. Any activated enzymes rapidly form an irreversible complex with inhibitor (tissue inhibitor of metalloproteinase; TIMP) and are not detected by APMA treatment (Galloway *et al.*, 1983). Similar activities have also been reported in the extracellular matrix (Poole *et al.*, 1974) and in the medium of cultured rabbit and human cartilage (Cartwright *et al.*, 1983; Murphy *et al.*, 1981). These secreted enzymes were not able to degrade proteoglycan in untreated culture medium. Increased release of proteoglycan from catabolin-stimulated articular cartilage is therefore not a tissue culture artefact caused by enzymes in the medium nibbling away at the matrix. Smaller degradation products were found in the medium of resorbing bovine nasal cartilage (Steinberg *et al.*, 1979; Dingle & Dingle, 1980) indicating that some active enzyme was secreted, possibly by the fibroblasts and endothelial cells of the vascular channels.

Any enzyme(s) capable of a restrictive cleavage must be exposed to the core protein for a very limited time. This could be achieved by rapid inhibition or by restricting access. If, for instance, the enzyme was immobilized or membrane-bound, after one clip the proteoglycan would diffuse out of the matrix, so preventing any further degradation. There is some evidence that enzymes involved in proteoglycan breakdown are not freely soluble (Dingle & Dingle, 1980). The cartilage matrix contains several classes of inhibitor which could limit the activity of serine-, cysteine- and metalloenzymes (Kuettner *et al.*, 1976; Roughley *et al.*, 1978; Sorgente *et al.*, 1975; Rifkin & Crowe, 1977; Knight *et al.*, 1979; Killackey *et al.*, 1983; Dean & Woessner, 1984). Enzymes such as cathepsin D are rapidly denatured above pH 5.5–6.0 (Dingle *et al.*, 1971; Woessner, 1973) and would be inhibited on diffusion away from the cell environment. The relative contribution of acid and neutral proteinases will depend on the local pH at the site where the initial clip occurs and this is not yet known.

The degradation and/or cleavage of the cartilage proteoglycan described here might also be caused by hydroxyl radicals. Phagocytic cells, or contact with immune complexes, produce free radicals (Halliwell, 1982) which can degrade hyaluronic acid by random cleavage of glycosidic bonds (Schmut & Hofmann, 1975; Cleland *et al.*, 1969)


Control
Depleted
V_0
V_t
(a)
(b)
(c)
(d)
$10^{-3} \times$ [^{35}S]Proteoglycan (d.p.m.)/fraction
$10^{-3} \times$ [^{35}S]Glycosaminoglycan (d.p.m.)/fraction
Fraction no.

and various glycoproteins by attacking the histidine residues (Greeth *et al.*, 1983). It is not known whether catabolin-stimulated chondrocytes can generate such radicals.

If catabolin is removed at any time from partially depleted cartilage and the explants are allowed to recover in control medium, increased loss of proteoglycans ceases within 24h. The effect

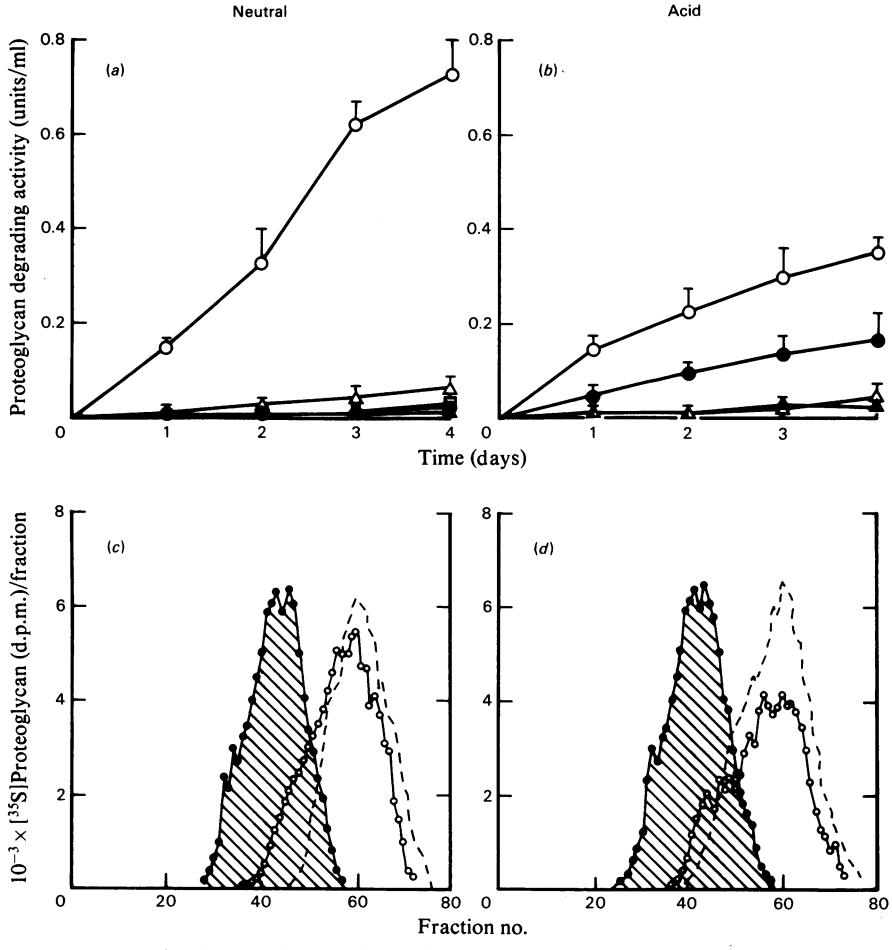


Fig. 8. *Cumulative release of proteinase activity into the medium*

The quantity and type of proteinase released into the culture medium was measured as described under 'Materials and methods' by using [^{35}S]proteoglycan trapped in polyacrylamide beads as a substrate. Medium from unlabelled control (filled symbols) and depleted (open symbols) cultures were incubated (*a*) at pH7.4 in the presence (●, ○) and absence (■, □) of APMA (0.7mM) and with APMA and 1,10-phenanthroline (2mM) (▲, △). Duplicate samples were assayed (*b*) at pH4.5 in the presence (▲, △) and absence (●, ○) of pepstatin (1 μg/ml). Values are the mean of six groups. Bars are S.E.M. The specificity of proteinase activity was assessed by using pooled conditioned medium from ^{35}S-labelled depleted cartilage containing the 'clipped monomers'. Samples were incubated (*c*) at pH7.4 in the presence of APMA and (*d*) at pH4.5 for 18h at 37°C. The digestion products (○—○) were mixed with proteinase inhibitors and eluted from a column of Sepharose CL-2B in the presence of 4M-guanidinium chloride. The starting material (shaded area) and a similar digest using trypsin (100 μg/ml) (---) are also shown.

Fig. 7. *Gel chromatography of the easily extracted and non-extractable proteoglycans from control and depleted cartilage*

(*a*) Samples of proteoglycan extracted with low ionic strength buffer were mixed with hyaluronic acid (2% on the basis of uronic acid content) and eluted from a column of Sepharose CL-2B. (*b*) Samples of proteoglycan extracted with low ionic strength buffer were eluted from a column of Sepharose CL-2B in the presence of 4M-guanidinium chloride. (*c*) Fractions (40–50) from (*a*) were pooled as indicated by the bar, digested with papain, and eluted from a column of Sephacryl S-200. (*d*) Material solubilized from the residues with papain was eluted from a column of Sephacryl S-200. ●, Control; ○, depleted.

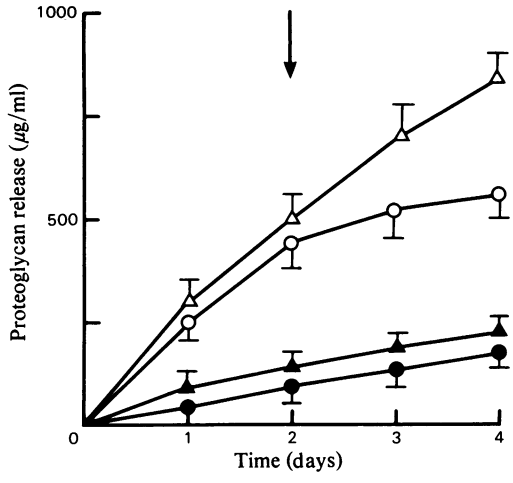


Fig. 9. *Release of proteoglycan during recovery*
Six paired cartilage halves were cultured with catabolin for 2 days. The partially depleted explants were washed and incubated for a further 2 days with control medium (○). The other halves were cultured in control medium for 4 days (●). Parallel cultures were grown for 4 days in the presence (△) or absence (▲) of catabolin. The data shown is cumulative dimethyl Methylene Blue reactive material in the medium as the mean of three experiments. Bars are S.E.M.

is therefore fully reversible. Knowledge of the mechanism which shifts chondrocyte metabolism towards net degradation of the matrix and conditions under which this type of control occurs would add greatly to our understanding of the pathological degradation of cartilage *in vivo*.

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