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Characterization of catabolin, the major product of pig synovial tissue that induces resorption of cartilage proteoglycan *in vitro*

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1. Pig synovium in organ culture produces material which induces living cartilage to resorb its proteoglycan *in vitro*. 2. The bioassay for this material was to measure glycosaminoglycan released from explants of bovine nasal-septal cartilage cultured for 8 days. The performance of the assay was greatly improved by adding cortisol succinate (0.1 μg/ml). This decreased the release of glycosaminoglycan from unstimulated cartilage without inhibiting its response to catabolic factors from the synovium. 3. By using this improved assay it was shown that 90% of the active materials in synovial culture medium were retained by dialysis membrane. 4. An active protein was partially purified from synovial culture medium by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography, gel filtration and preparative isoelectric focusing. 5. This protein, called catabolin, had mol.wt. 17000 and pI 4.6. 6. Synovial culture medium concentrated in dialysis tubing was subjected to gel chromatography and found to contain one major active component, which was eluted at the same position as the partially purified catabolin. 7. The synovial culture medium was not inactivated by heating (70°C for 10 min), nor were diluted preparations of partially purified catabolin, but concentrated crude preparations were thermolabile. 8. These results suggest that catabolin is the major substance produced by the synovial tissue in culture which induces resorption of proteoglycan of living cartilage *in vitro*. 9. Other cultured soft connective tissues produced catabolin-like activity. The example of sclera is shown, and production was inhibited by cortisol succinate (0.1 μg/ml). 10. It is suggested that catabolin may be a general product of soft connective tissues in culture, and its physiological function may be to induce resorption of connective-tissue matrix after injury.

The cellular and molecular mechanisms of the breakdown of connective-tissue matrices in physiological and pathological states are obscure. One approach to these problems is to study matrix resorption in organ culture. Cartilage is highly suitable for this purpose because it is a relatively simple tissue, consisting only of chondrocytes and two major matrix components (i.e. proteoglycan and collagen). Moreover, to study resorption of cartilage matrix is relevant to pathology, because loss of articular cartilage is the major problem of arthritic diseases. After the observation by Fell & Jubb (1977) that synovial tissue in organ culture would induce degradation of living cartilage, it was shown that the synovial tissue produced an acidic protein of about 20000 molecular weight which caused chondrocytes to resorb the proteoglycan of the cartilage matrix (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980). This protein we called 'catabolin', although it was not possible to determine whether it was the major active component of the synovial culture medium. We estimated that only about 20–30% of the catabolic activity of the conditioned medium was recoverable by the $(NH_4)_2SO_4$ fractionation that was used to identify catabolin (Saklatvala & Dingle, 1980).

Attempts to purify catabolin and to assess its overall contribution to the activity of the synovial medium have been hindered by the variable behaviour of explants of bovine nasal cartilage on which catabolic activity was assayed. The assay (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980) involved measuring the amount of glycosaminoglycan released from explants of bovine nasal cartilage maintained for 8 days in culture, but about one experiment in four was difficult to interpret because of the high release of glycosaminoglycan from control explants. In this paper I describe how

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the release of glycosaminoglycan from the control explants could be suppressed by including cortisol succinate in the culture medium. This greatly improved the performance of the assay, since the steroid did not hinder induction of glycosaminoglycan release from the cartilage caused by either the synovial culture medium or catabolin-containing fractions derived from it.

The improved assay gave much clearer results with fractions from chromatography and other separation procedures, and on the basis of the experiments described below it is suggested that most of the proteoglycan resorption induced by the synovial culture medium can be attributed to catabolin. Preparative isoelectric focusing showed two species of catabolic protein, a major one of pI 4.6 and a minor one of pI 5.1.

Catabolin-like activity is produced in organ culture by several types of soft connective tissue. If catabolin is a general product of damaged connective tissue, then it may have a function *in vivo* in the reaction of connective tissues to injury. Its relationship to other 'factors' thought to be involved in connective-tissue catabolism is discussed.

Materials and methods

Materials

Dulbecco's (Vogt & Dulbecco, 1960) modification of Eagle's culture medium (DME medium) was obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Papain (type III), whale chondroitin sulphate (grade III), cortisol succinate and protein standards for chromatography were from Sigma. 1,9-DimethylMethylene Blue was from Serva Feinbiochemica, Heidelberg, Germany. Carbowax 20M (polyethylene glycol) was from Raymond A. Lamb, London N.W.10, U.K. Ultrogel AcA54 was from LKB Instruments, Croydon, Surrey, U.K. DEAE-cellulose (DE-52) was from Whatman, Springfield Mill, Maidstone, Kent, U.K. Sephadex-IEF and Pharmalyte carrier ampholytes for isoelectric focusing were from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were analytical-reagent or best available grade.

Culture of synovium

The soft tissue lining was dissected from the metacarpophalangeal joints of young adult pigs within 2h of the animals' slaughter. This tissue, subsequently referred to as synovium, was cut into pieces of about 0.5 cm^3 and cultured in DME medium at 4g of tissue/100ml at 37°C in CO_2/air (1:19). The medium contained penicillin (100i.u./ml) and streptomycin (100μg/ml). After 24h the medium was changed and the used medium was discarded. Subsequently the medium was changed at 2–3-day intervals up to 8 days in culture; the used medium was pooled for further study.

Culture of sclera

Fresh bovine sclera was dissected free of conjunctiva and scraped free of choroid. It was cut into strips 2mm × 2mm × 5mm and cultured at 8g/100ml of medium exactly as described for synovial tissue.

$(NH_4)_2SO_4$ fractionation

For experiments on the effects of cortisol succinate on bovine nasal cartilage a 60–95%-satd.-$(NH_4)_2SO_4$ fraction of pig synovial medium was prepared. Solid $(NH_4)_2SO_4$ was dissolved in medium at 4°C to 60% saturation. After standing for 1h, the mixture was centrifuged at 10000rev./min (12000*g*) for 30min. The supernatant was decanted and solid $(NH_4)_2SO_4$ added to give 95% saturation. The mixture was left for 1h and centrifuged as before. The pellet was dissolved in 0.01 of the original volume of phosphate-buffered saline (1mM-NaH_2PO_4/18mM-Na_2HPO_4/145mM-NaCl, pH 7.4) and dialysed against it before use. For ion-exchange chromatography the synovial medium was adjusted directly to 95% saturation at 4°C with solid $(NH_4)_2SO_4$. After standing for 1h it was centrifuged at 10000rev./min for 30min. This precipitate was dissolved in 0.01 of the original volume and dialysed against 20mM-glycine/NaOH buffer, pH 8.5.

Culture of bovine nasal cartilage

This was carried out essentially as described by Dingle *et al.* (1979). Discs of cartilage (0.4cm diameter × 0.1cm thick) were cut from the nasal septum of recently slaughtered animals. Each disc was placed on a stainless-steel grid in a compartment of a 100mm-square Petri dish containing 25 compartments; 1.2ml of DME medium containing 5% normal sheep serum (heat-inactivated for 30min at 56°C) was added to each compartment. For some experiments other concentrations and types of serum were used. The medium contained penicillin (100i.u./ml) and streptomycin (100μg/ml). The discs were maintained at 37°C in CO_2/air (1:19). Samples to be tested for activity on the cartilage were incorporated in the culture medium, and each was assayed on five discs in separate compartments. After 4 days the medium was replaced (the fresh medium contained the sample under test), and the culture was ended after another 4 days (total culture period 8 days). The glycosaminoglycan content of the discs and of the culture medium was measured, and the results were expressed as the percentage of total glycosaminoglycan (medium + tissue) released into the medium during the 8 days of culture.

Assay of glycosaminoglycan

To each disc was added 14 μg of papain in 0.5 ml of 0.05 M-phosphate buffer containing 1 mM-*N*-acetylcysteine and 1 mM-EDTA, pH 6.5. The discs were digested for 2 h at 65°C. This digest, and the samples of culture medium, were assayed for glycosaminoglycan with the metachromatic dye dimethylMethylene Blue by a modification of the method of Humbel & Etringer (1974). 1,9-Dimethylmethylene Blue (16 mg) was dissolved in 100 ml of 0.1 M-formate buffer, pH 3.5, containing 5% (v/v) ethanol. This solution was diluted 10-fold with the formate buffer for use. The sample (10–40 μl) was added to 5 ml of dye and the A_{535} was measured within 5 min. The working range of the assay was an A_{535} increase up to 0.2. Samples containing serum took up to 1 min to equilibrate with the dye. Serum at final concentrations greater than 0.2% lowered the absorbance change; the highest final concentration of serum used was 0.04%. A standard curve from 5 to 30 μg of whale chondroitin sulphate was included with each set of assays, and the glycosaminoglycan content of the samples was expressed as μg of chondroitin sulphate.

Other methods

Protein concentration was measured by the method of Lowry *et al.* (1951). Uronic acid was measured by the carbazole method (Bitter & Muir, 1962). Dialysis tubing was acetylated before use by immersing 18/32-in Visking tubing in pyridine containing 25% (v/v) acetic anhydride for 20 h at room temperature. The tubing was then washed exhaustively before use (after acetylation the tubing was impermeable to substances of >1500 mol.wt.). Isoelectric focusing in polyacrylamide gel was done as previously described (Saklatvala & Dingle, 1980). Preparative isoelectric focusing in Sephadex slabs was performed with Pharmalyte carrier ampholytes according to the makers' instructions. Details of this and chromatography are given in the appropriate Figure legends.

Results

Effect of cortisol succinate on the release of glycosaminoglycan from explants of cartilage

Table 1 shows the variability of the percentage of glycosaminoglycan released by explants of seven different nasal septa cultured in the absence or presence of cortisol succinate. Without the steroid the values ranged from 12 to 61%, and were lowered in the presence of steroids to 12 to 35%.

In practice, experiments done with nasal septa 1–4, without steroid, would have been difficult to interpret because of the high control release values, whereas those done with nasal septa 5–7 would have given satisfactory results. In the presence of the steroid all the nasal septa (except possibly no. 4) showed acceptable control values for glycosaminoglycan release.

Effect of different sera on glycosaminoglycan release from explants of cartilage

In initial experiments (results not shown) it was found that the percentage of glycosaminoglycan released into the medium from control explants of cartilage was not affected by serum concentration (5, 10 or 15%) and was the same whether the serum was from sheep, rabbit or foetal calf. All sera were heat-inactivated (56°C for 30 min) before they were used in organ culture.

Effect of cortisol succinate on the glycosaminoglycan content of the cartilage explants

Table 2 shows the results of experiments on cartilage from three of the animals shown in Table 1, comparing the amount of glycosaminoglycan per explant before culture with the amount present in the explant and medium after culture. Cartilage from nasal septum 6, which released an acceptable amount of its glycosaminoglycan (18%; see Table 1), showed no net synthesis or loss of glycosaminoglycan when cultured in the absence or presence of cortisol succinate. Nasal septa 3 and 4 gave relatively high glycosaminoglycan release values (36 and 61%; see Table 1), and in the absence of steroid there was a slight overall loss of glycosaminoglycan during the culture period. Table 2 also shows that the type of serum used (sheep, rabbit or foetal calf) had no effect on the overall glycosaminoglycan content. It was concluded that in the presence or absence of the steroid there was no measurable net synthesis of glycosaminoglycan over the culture period, and that its inclusion in the assay

Table 1. *Effect of cortisol succinate on the percentage of glycosaminoglycan released by bovine nasal cartilage in culture*
The amount of glycosaminoglycan released into the culture medium over 8 days is expressed as a percentage of total glycosaminoglycan. Each value is the mean ± S.E.M. for 15 separate disc cultures. Data are shown for septa from seven different animals; —, not measured.

Bovine nasal septum no. ...	1	2	3	4	5	6	7
In 5% sheep serum	29 ± 3	30 ± 5	36 ± 3	61 ± 8	20 ± 1	18 ± 1	12 ± 1
In 5% sheep serum + cortisol succinate (1 μg/ml)	—	18 ± 1	17 ± 1	31 ± 3	16 ± 1	—	12 ± 1
In 5% sheep serum + cortisol succinate (0.1 μg/ml)	14 ± 1	15 ± 2	20 ± 1	35 ± 2	—	13 ± 2	—

Table 2. *Change of glycosaminoglycan content of bovine nasal cartilage during culture*
The amounts of glycosaminoglycan (μg) are means ± S.E.M. for 15 discs. The percentages of glycosaminoglycan released into the medium are shown in Table 1. All sera were heat-inactivated before use (56°C for 30 min).

Sample	Serum content	Cortisol succinate (μg/ml)	Nasal septum 3	Nasal septum 4	Nasal septum 6
Cartilage disc before culture	—	—	1675 ± 106	1854 ± 107	2090 ± 78
Cartilage disc + medium after 8 days	5% sheep	0	1006 ± 73	1185 ± 125	2310 ± 189
Cartilage disc + medium after 8 days	5% sheep	0.1	1387 ± 59	1268 ± 143	—
Cartilage disc + medium after 8 days	5% sheep	1.0	1105 ± 110	1554 ± 85	2166 ± 198
Cartilage disc + medium after 8 days	10% sheep	0	—	—	2299 ± 147
Cartilage disc + medium after 8 days	5% foetal calf	0	1142 ± 69	—	—
Cartilage disc + medium after 8 days	5% rabbit	0	—	—	2050 ± 181
Cartilage disc + medium after 8 days	10% rabbit	0	—	—	2178 ± 178

had no effect on the overall amount of glycosaminoglycan recovered from the culture.

Effect of cortisol succinate on the release of glycosaminoglycan from cartilage explants stimulated by products of synovium in organ culture

Fig. 1(*a*) illustrates the effect of 1 μg of cortisol succinate/ml on the release of glycosaminoglycan from explants of the bovine nasal cartilage stimulated by different amounts of the $(NH_4)_2SO_4$ fraction made from synovial culture medium. The steroid depressed the release from both the unstimulated and stimulated explants by the same amount, but it did not alter the slope of the dose–response curve. Cortisol succinate at 0.1 μg/ml also had no effect on the dose–response curve (Fig. 1*b*). However, at either 1 μg/ml or 0.1 μg/ml the steroid inhibited slightly the release of glycosaminoglycan induced by the conditioned synovial medium from which the $(NH_4)_2SO_4$ fraction was made (Fig. 1*c*). In other experiments (results not shown) the inhibition was more marked at 1 μg of cortisol succinate/ml, but at 0.1 μg/ml the slope of the dose–response curve was never decreased by more than 30%. Since the steroid decreased the release of glycosaminoglycan from unstimulated cartilage, but caused little decrease in the slope of the dose–response curve of either the synovial conditioned medium or the $(NH_4)_2SO_4$ fraction prepared from it, it was decided that cortisol succinate in the culture medium at 0.1 μg/ml would improve the assay without inhibiting any active components.

Effect of dialysis on the activity of synovial conditioned medium

As a preliminary to further experiments on the conditioned synovial medium, the effect of dialysing it was investigated. Two types of experiment were done. In the first the conditioned medium was dialysed for 48 h against an equal volume of unused medium. The solutions inside and outside the dialysis bag were tested for activity against bovine nasal cartilage. Little active material was found outside the bag (Fig. 2). At the highest dose there was significant release of glycosaminoglycan induced by products that had passed through the dialysis membrane, although this was less than that caused by the lowest dose of the retained materials. It was calculated that 90% of the active substances were retained. In the second experiment the synovial medium was dialysed against a large volume. The activity of the medium before and after such exhaustive dialysis was measured (Fig. 2). There was no detectable loss of activity as a result of dialysis. Taken together, these results showed that (*a*) there was little catabolic activity induced by low-molecular-weight substances and (*b*) low-molecular-weight substances were not required to enhance the effect of active components of high molecular weight (there being no diminution of activity after dialysis against a large volume), so it was concluded that all the substances responsible for inducing glycosaminoglycan release from the cartilage were retained by the dialysis tubing.

Chromatography of cartilage-catabolic factor

Protein was precipitated from synovial medium by adjusting it to 95% saturation with $(NH_4)_2SO_4$ as described in the Materials and methods section. By comparing dose–response curves for the original medium and the precipitated protein it was calculated that only about 20–30% of the active material was recovered in the precipitate. This protein fraction had a high content of hyaluronic acid (about 1 mg of uronic acid/5 mg of protein), which prevented good resolution on gel filtration, and so a stepwise procedure with DEAE-cellulose was used to remove the hyaluronic acid (Fig. 3). The salt fraction was applied to the column at pH 8.5 in low-ionic-strength buffer, the bed was washed with 3 vol. of the buffer and was eluted successively with buffer containing 0.13 M-NaCl and 0.5 M-NaCl.

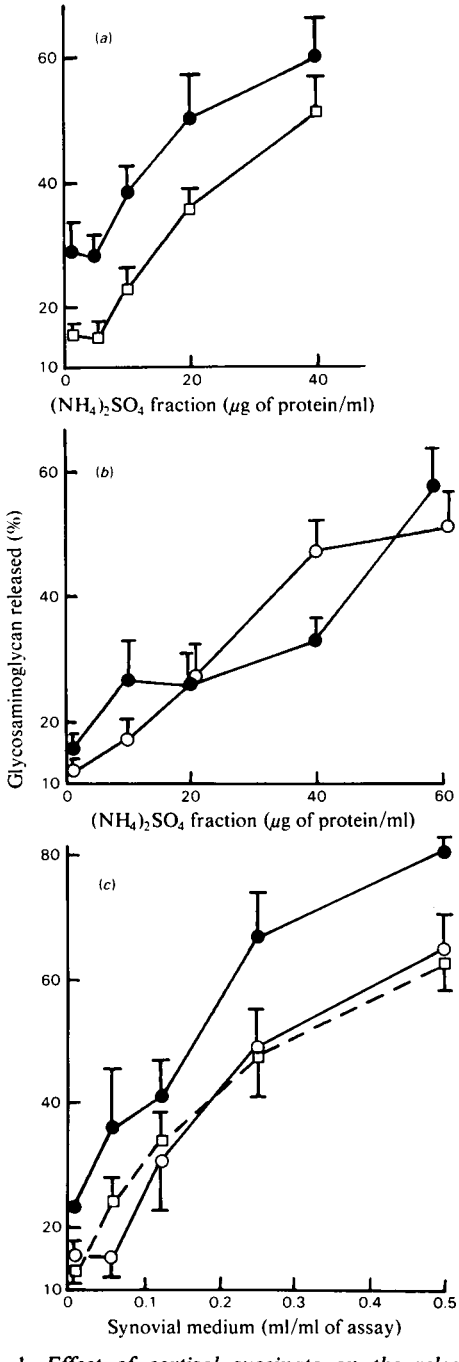

Fig. 1. *Effect of cortisol succinate on the release of cartilage glycosaminoglycan induced by synovial culture products*

(*a*) Glycosaminoglycan released (%) from bovine nasal-cartilage explants by increasing amounts of the 60–95%-satd.-$(NH_4)_2SO_4$ fraction of pig synovial culture medium in the absence (●) and presence (□) of cortisol succinate at 1 μg/ml. (*b*) As (*a*), except cortisol succinate at 0.1 μg/ml (○). (*c*) As (*a*) and (*b*), except unfractionated synovial culture medium was used rather than $(NH_4)_2SO_4$ fraction. Assays were done without (●) and with added cortisol succinate at (□) 1 and (○) 0.1 μg/ml. The bars are S.E.M. for quintuplicate assays.

After concentration and dialysis against phosphate-buffered saline, the eluates were assayed on bovine nasal cartilage. Active material was present in the fraction eluted by 0.13 M-NaCl; none was present in the 0.5 M-NaCl eluate. Hyaluronic acid (estimated as uronic acid) was in the 0.5 M-NaCl eluate. Recovery of activity from this step has varied from 40 to 70%. The concentrated 0.13 M-NaCl eluate was chromatographed on Ultrogel AcA54, and the fractions were assayed for activity against bovine nasal cartilage (Fig. 4*a*). The assay done in the presence of steroid gave low control values, and the active

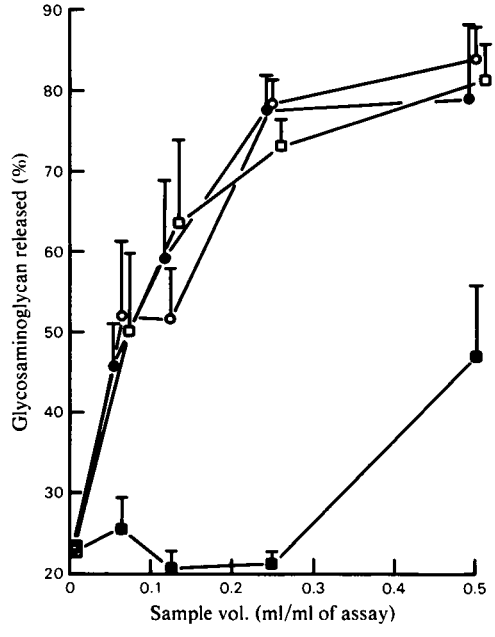

Fig. 2. *Cartilage-catabolic activity of synovial medium after dialysis*

The effect of synovial culture medium was assayed on nasal cartilage at increasing concentrations (○). A sample was dialysed against an equal volume of unused DME medium for 48 h, and the residue inside the dialysis bag (□) and the medium outside the bag (■) were assayed. Another sample of synovial medium was assayed after dialysis for 48 h against a large volume of unused DME medium (●). All cartilage assays were done in the presence of cortisol succinate (0.1 μg/ml). The bars are S.E.M. for quintuplicate assays.

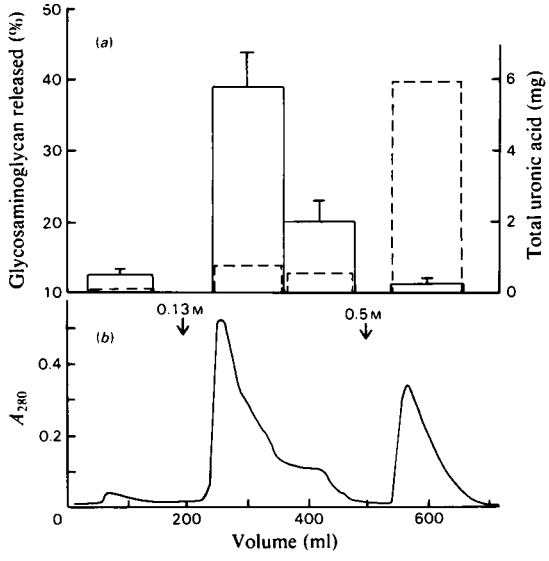

Fig. 3. *DEAE-cellulose chromatography of synovial culture material*

Protein (55 mg) obtained from 2 litres of synovial conditioned medium by $(NH_4)_2SO_4$ precipitation was dialysed against 20 mM-glycine/HCl buffer, pH 8.5, and pumped on to a column of DEAE-cellulose (3 cm × 8 cm). The column was eluted successively with buffer containing 0.13 M- and 0.5 M-NaCl as shown by the arrows in part (*b*). Fractions pooled as indicated by the blocks in part (*a*) were concentrated 10-fold, dialysed against phosphate-buffered saline and assayed at 8 μl/ml against bovine nasal cartilage with cortisol succinate at 0.1 μg/ml (unbroken line blocks show glycosaminoglycan release; bars are S.E.M.). Hyaluronate (as uronic acid) is shown by the broken line blocks.

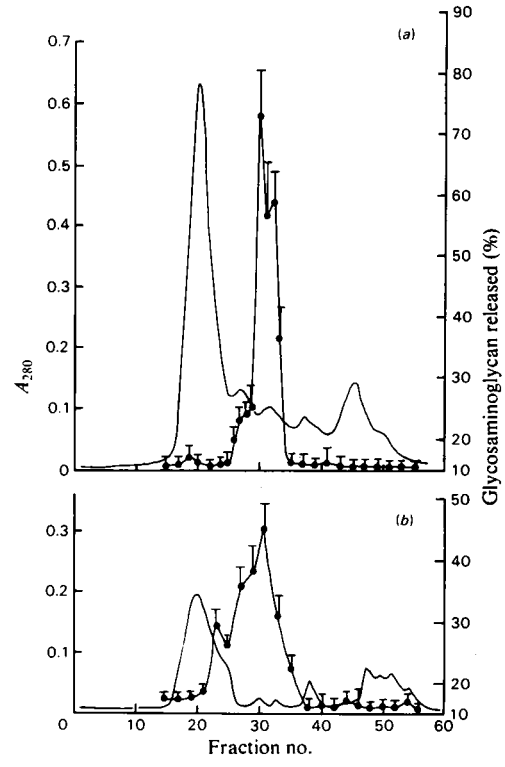

Fig. 4. *Gel filtration of (a) active material from DEAE-cellulose chromatography and (b) concentrated synovial culture medium*

(*a*) A 2.5 ml sample (20 mg of protein) of the 0.13 M-NaCl eluate from DEAE-cellulose chromatography (Fig. 3) was chromatographed on a column of Ultrogel AcA54 (1.5 cm × 90 cm) at a flow rate of 10 ml/h. The column buffer was phosphate-buffered saline; 3.3 ml fractions were collected and assayed on bovine nasal cartilage at 60 μl/ml (in the presence of cortisol succinate, 0.1 μg/ml). ●, Glycosaminoglycan release (%) (bars show S.E.M.); ——, A_{280} (protein). (*b*) Synovial culture medium was concentrated 30-fold in dialysis tubing by Carbowax and 2.5 ml was chromatographed and assayed as in (*a*). The column was calibrated with Blue Dextran, bovine serum albumin, carbonic anhydrase, soya-bean trypsin inhibitor, cytochrome *c* and aprotinin (Trasylol).

fractions were unambiguously defined. There was one active component which was eluted at a position corresponding to 16000–18000 mol.wt. The recovery of activity was 70–80%. We have previously shown that this component has pI 4.6, and called it catabolin (Saklatvala & Dingle, 1980). Because only about 20% of the activity of the synovial conditioned medium was recovered by the $(NH_4)_2SO_4$ step, it was possible that catabolin was a minor active component of the synovial culture medium. Since all the active material of the medium was retained by the dialysis membrane, the medium was concentrated 30-fold in dialysis tubing surrounded by Carbowax, dialysed against the column buffer and chromatographed on the same column of Ultrogel AcA54 (Fig. 4*b*). One peak of active material was observed, and its elution position corresponded exactly to the peak seen in Fig. 4(*a*). The peak was slightly broader, but that may have been due to the viscous nature of the sample affecting the resolution (hyaluronic acid had not been removed from the sample). There was no evidence of any other major active component, and the recovery was about 70%. Material from the active peak was subjected to isoelectric focusing in polyacrylamide gels, which were sliced, eluted and assayed for activity. A major active component was identified at pH 4.5–4.7 (results not shown; recovery about 20%).

Thermal inactivation of catabolin

Fractionation of the synovial medium concentrated by dialysis membrane showed an active component of similar size and isoelectric point to that found in the $(NH_4)_2SO_4$ fraction. Previously, the active material of the $(NH_4)_2SO_4$ fraction was shown to be thermolabile (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980); therefore if the activity of synovial medium was largely due to catabolin it too should have been destroyed by heating. In fact the activity of the medium was only slightly diminished by heating. The dose–response curve for synovial medium heated at 70°C for 10min was only slightly lower than that obtained with unheated medium (Fig. 5*a*). To test whether the catabolin in the synovial medium was being inactivated by heating, a 0–95%-saturated $(NH_4)_2SO_4$ fraction was prepared from heated and unheated medium. The fraction made from the heated medium was only slightly less active, and it was clear that the active substance in the synovial medium was not being inactivated as expected (Fig. 5*b*). A further experiment was done in which samples of the $(NH_4)_2SO_4$ fraction were (*a*) heated and then diluted 1 in 50 (i.e. to about the concentration expected in synovial medium) for assay in organ culture, (*b*) heated after dilution and (*c*) assayed without heating. The samples heated before dilution lost activity as expected (Fig. 5*c*), but the sample heated after dilution was still active. The crude catabolin was therefore thermostable in dilute solution, but thermolabile when concentrated, and so the stability of activity of the synovial culture medium to heating was consistent with it being attributed to catabolin. Such concentration-dependence implies that the thermolability may really be an enzymic inactivation.

Preparative isoelectric focusing

Active material from chromatography on Ultrogel AcA54 was subjected to isoelectric focusing in a thin layer of Sephadex. Fig. 6 shows that the main active component was found at pH4.6–4.8, and there was a smaller peak at pH5.1. This second active component had not been detected on analytical focusing in polyacrylamide gels. The main peak was chromatographed on Ultrogel AcA54 (Fig. 7), and the active material was eluted at a position corresponding to 17000–18000mol.wt. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the fractions showed the presence of several proteins in the mol.wt. range 12000–25000 (results not shown). Recovery of activity from preparative isoelectric focusing was only about 10%.

Catabolin-like activity from other connective tissues: bovine sclera

Connective tissues other than synovium produce cartilage-catabolic activity in organ culture. Joint capsule, sub-dermal tissue, cornea and sclera have been found to produce catabolin-like activity. Fig. 8 shows production by explants of bovine sclera. The scleral conditioned medium was assayed on the cartilage at two doses. No active material was released by scleral explants which had first been killed by freezing and thawing (four times). Production of activity was substantially inhibited by cortisol succinate at 0.1 μg/ml.

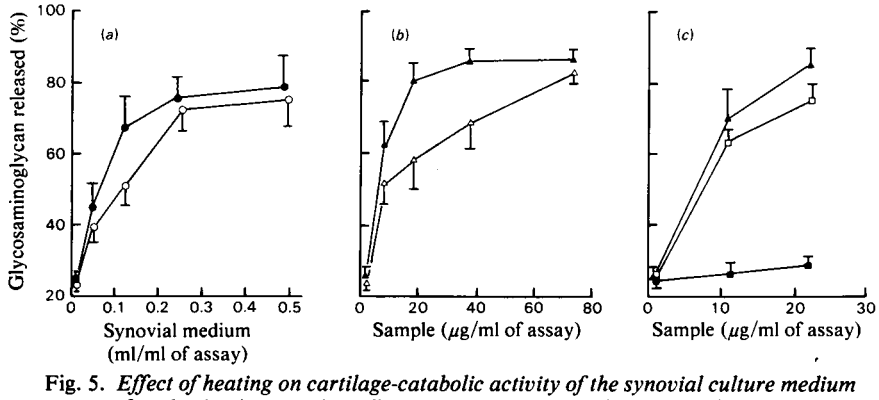

Fig. 5. *Effect of heating on cartilage-catabolic activity of the synovial culture medium*

Dose–response curves for the bovine nasal-cartilage assay are shown for heated (70°C, 10min) and unheated samples of synovial culture medium and the 0–95%-satd.-$(NH_4)_2SO_4$ fraction made from it. The assays were done in the presence of cortisol succinate (0.1 μg/ml). The bars represent S.E.M. (*a*) Unheated synovial medium (●); heated synovial medium (○). (*b*) 0–95%-satd.-$(NH_4)_2SO_4$ fractions made from unheated (▲) and heated (△) synovial medium. (*c*) 0–95%-satd.-$(NH_4)_2SO_4$ fraction was assayed before heating (▲), after heating in concentrated form (■), and after heating when diluted 1:50 into culture medium (□).

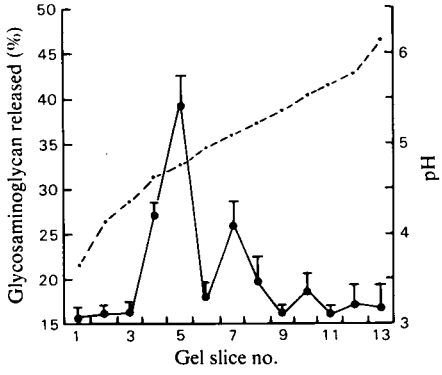

Fig. 6. *Preparative isoelectric focusing of active synovial material after ion-exchange and gel chromatography*
The sample was prepared as follows: $(NH_4)_2SO_4$ fraction from 8 litres of synovial medium was chromatographed on DEAE-cellulose (as in Fig. 3), and active material (the 0.13 M-NaCl eluate) was concentrated to 8 ml (70 mg of protein) and chromatographed on Ultrogel AcA54 as in Fig. 4, except that a larger column (4.4 cm × 100 cm) was used. Active fractions were pooled, concentrated and dialysed against 1% glycine. This sample (5 mg of protein) was made up in Sephadex-IEF to give a 20 ml slurry containing 1 ml of pH 4–6.5 Pharmalyte solution. The slurry was spread as a thin layer on a glass plate (11.5 cm × 23 cm). Electrode wicks contained 0.1 M-H_3PO_4 (anode) and 0.1 M-NaOH (cathode), and focusing was carried out at 8 W constant power for 5 h. The slab was sectioned and the slurries were eluted with 2.0 ml of water (for pH measurement) and then 2.0 ml of phosphate-buffered saline. The eluates (4 ml) were assayed at 12 μl/ml on bovine nasal cartilage in the presence of cortisol succinate (0.1 μg/ml). –·–·, pH; ●, glycosaminoglycan released (%) (bars represent S.E.M.).

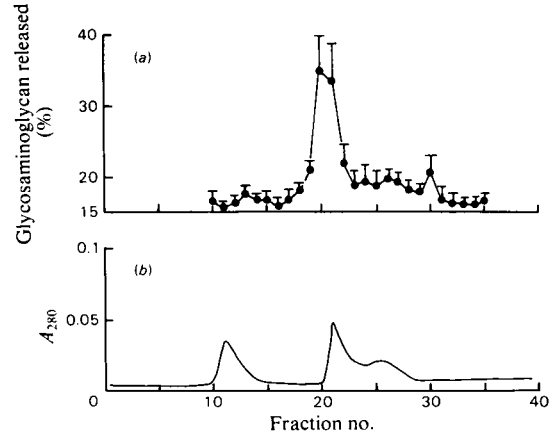

Fig. 7. *Gel chromatography of pI-4.6 peak from isoelectric focusing*
The major active peak from isoelectric focusing (fractions 4 and 5 in Fig. 6) were concentrated to 2 ml in dialysis tubing by Carbowax, dialysed against phosphate-buffered saline and chromatographed on a column (1.5 cm × 90 cm) of Ultrogel AcA54 as in Fig. 4, except that fractions were 4.8 ml. These were assayed on bovine nasal cartilage at 50 μl/ml (*a*). Protein-elution profile is shown as A_{280} (*b*).

Discussion

In order to establish a more reliable and precise bioassay for cartilage degradation, it was necessary to include cortisol succinate in the culture medium. The steroid lowered the release of glycosaminoglycan from unstimulated cartilage, but had no effect on the total glycosaminoglycan recovered from cartilage cultures. Many workers have studied the behaviour of unstimulated cartilage explants, and the reported amounts of glycosaminoglycan released differ widely. Values vary from as high as 60% released from rabbit articular cartilage over 4 days (Sandy *et al.*, 1978) and 50% from rat costal cartilage over 10 days (Wasteson *et al.*, 1972) to 20% from bovine nasal cartilage over 8 days (Dingle *et al.*, 1979; Steinberg *et al.*, 1979*a*; Saklatvala & Dingle, 1980). The results reported here show that bovine nasal cartilage from different animals released widely differing amounts of glycosaminoglycan in unstimulated explant culture. It is not known to what extent this variable release is due to (*a*) leaching of proteoglycan, (*b*) failure of newly synthesized proteoglycan to become trapped in the matrix, and (*c*) degradation of existing proteoglycan. Nor is it clear how the steroid suppresses this release. The finding of no net synthesis of glycosaminoglycan (in the absence of steroid) was in agreement with the observations of Benya & Nimmi (1979), but not with those of Steinberg *et al.* (1979*a*), who found increases of up to 70% in the total glycosaminoglycan content of bovine nasal explant cultures. Different types and concentrations of serum were without effect on the total glycosaminoglycan recovered in the present study, and the reason for the discrepancy is not understood.

Inclusion of the steroid in the culture medium did not inhibit the response of the cartilage to the synovial products, and it greatly improved the performance of the assay (*a*) by increasing the ratio of active to control (by suppressing the controls), (*b*) by improving reproducibility and (*c*) by making all the nasal septa give interpretable results. By using this modified assay, it was possible to show that 90% of the catabolically active products of synovial culture were retained by the dialysis membrane and that catabolin was the major active product in the

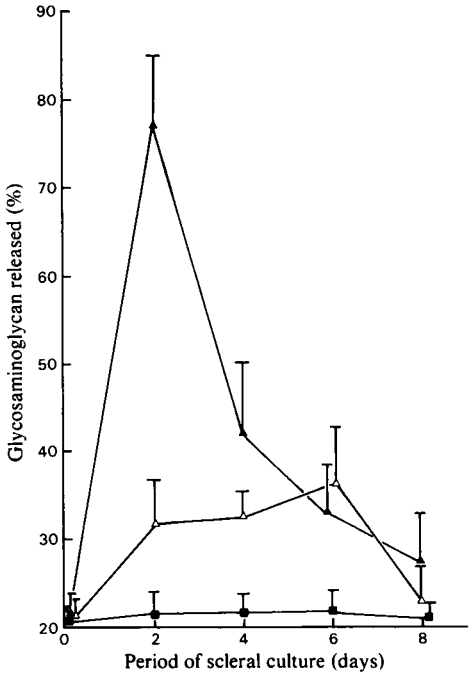

Fig. 8. *Production of cartilage-catabolic activity by bovine sclera*

Bovine sclera was cultured for 8 days as described in the Materials and methods section. Medium was changed every 2 days and samples of this were assayed at 1:4 dilution in the bovine nasal-cartilage assay (in the presence of cortisol succinate, 0.1 μg/ml). The Figure shows sclera cultured without (▲) and with (△) cortisol succinate (0.1 μg/ml) and after freezing and thawing four times immediately after dissection (■).

synovial culture medium. The improved assay permitted more accurate determination of the molecular weight of catabolin (17000–18000, compared with our previous estimate of 20000: Saklatvala & Dingle, 1980), and on preparative isoelectric focusing it revealed the presence of a second active component of pI 5.1 in addition to the major one of pI 4.6. This second component could be another isoelectric form of the major component, or it might be another polypeptide. It accounted for about 20% of the activity recovered from isoelectric focusing. Provisionally they could be called catabolins I (pI 4.6) and II (pI 5.1).

Although the thermostability of the active component in the synovial medium at first suggested that most of the activity might be due to a factor other than catabolin, it was found that the crude catabolin preparations were thermostable when dilute, although thermolabile when concentrated. Probably the thermolability previously described (Dingle *et al.*, 1979) was the result of enzymic inactivation rather than thermal denaturation.

Catabolin is therefore apparently the major product of the synovial tissue in organ culture which induces degradation of the proteoglycan matrix of the cartilage, and the poor recovery of catabolin on $(NH_4)_2SO_4$ fractionation (about 20%) was likely to be due to its very low concentration in the synovial medium.

The relationship of catabolin to other 'factors' or 'cytokines' proposed to regulate connective-tissue catabolism is not yet clear. A substance (or substances) from human synovium in organ culture stimulates production of prostaglandin and plasminogen activator by human chondrocytes in culture (Meats *et al.*, 1980). Cultured chondrocytes are stimulated to produce collagenase by a protein fraction (corresponding to about 14000 mol.wt.) obtained from endotoxin-stimulated macrophages (Deshmukh-Phadke *et al.*, 1980; Ridge *et al.*, 1980) and monocytes (Phadke *et al.*, 1981). This factor may be the same monocyte product (monokine) as that originally described by Dayer *et al.* (1979), which stimulates synovial cells to secrete collagenase, and which is now thought to be identical with the monokine interleukin 1 (Dayer *et al.*, 1981; Mizel *et al.*, 1981). Of these various factors, only interleukin 1 has been well characterized. It was previously called lymphocyte-activating factor (LAF; see Aarden *et al.*, 1980). Mouse interleukin 1 is a protein of pI 5.0 and 14000 mol.wt. (Mizel & Mizel, 1981), and the human equivalent is of similar size but of pI 6.5 (Wood, 1979), so it appears to be a less acidic protein that catabolin. Furthermore, there is no evidence that catabolin causes matrix resorption by stimulating chondrocytes to secrete proteinases, although the hypothesis is attractive.

Tyler *et al.* (1981) have found that cortisol succinate at 0.1 μg/ml substantially inhibited the release of cartilage-catabolic activity from cultured pig synovium. Their results, taken together with those presented here, show that although cortisol succinate has little effect on the action of catabolin it markedly inhibits its production by the soft connective tissue. These findings are consistent with those of Steinberg *et al.* (1979*b*), who observed that cortisol lowered the glycosaminoglycan release from bovine nasal cartilage cultured alone and when co-cultured in contact with human rheumatoid synovium.

Sclera and other soft connective tissues were found to produce catabolin, and the production was suppressed by cortisol succinate (0.1 μg/ml). Therefore, to release catabolin may be a quite general property of soft connective tissues in culture. Cartilage does not produce catabolin, but does respond to it, and so is an ideal tissue for its assay.

The failure of cartilage to produce catabolin may be related to its having only one cell type (the chondrocyte). A plausible hypothesis of the function of catabolin is as follows. It is made by cells in the connective tissue in response to injury and it causes the cells to resorb their matrix. Such resorption necessarily precedes the proliferation of fibroblastic and endothelial cells during the formation of granulation tissue. Studies with purified catabolin may reveal whether or not it is a general chemical signal to stimulate connective tissues to resorb.

In conclusion, it is suggested that 'catabolins' should be defined as proteins of molecular weight 15000–25000 produced in tissue culture, which induce cartilage resorption *in vitro*. This loose definition should suffice until the molecules are isolated and their action on connective tissues is better understood.

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