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# A Cartilage Catabolic Factor from Synovium

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Porcine synovium in organ culture produces a factor that causes chondrocytes to degrade their matrix. A quantitative assay for the factor, for which the cartilage of bovine nasal septum is used, is described. Evidence is presented that the catabolic factor is a protein.

Previous experiments (Fell & Jubb, 1977) have shown that, when porcine synovium and articular cartilage were cultured apart in the same dish, the matrix of the cartilage was depleted, but if the cartilage was killed before culture, the matrix was unaffected. It seemed possible that synovium produces a catabolic factor that causes chondrocytes to degrade cartilage matrix. The first experiments shown below were made to discover whether such a substance existed, and subsequently, because of the putative factor's potential importance in cartilage matrix regulation, the initial stages in its isolation and characterization are reported.

## Materials and Methods

Dulbecco's (Vogt & Dulbecco, 1960) modification of Eagle's culture medium (DME medium) was used. Chick ovoinhibitor was purified as described by Barrett (1974). Bovine trypsin (type I), α-chymotrypsin (type I-S), papain (type III) and shark chondroitin sulphate (grade III) were obtained from Sigma; Sepharose 4B was obtained from Pharmacia, Ultrogel AcA54 from LKB, and 1,9-dimethyl Methylene Blue from Serva Feinbiochemica.

### *Culture of porcine synovium and cartilage*

Pieces of synovial tissue (0.5 $cm^3$) dissected from the metacarpophalangeal joints of adult pigs were cultured in DME medium at 37°C in an atmosphere of $CO_2$/air (1:19). The medium was changed after the first day and then every 2 days up to 14 days. Medium from the first change was generally discarded; the rest was pooled and deep-frozen to await fractionation.

Paired explants of articular cartilage were prepared from the metacarpals of young pigs (5–9 months old) and were cultured as described previously in DME medium with 15% (v/v) heat-inactivated normal rabbit serum (Fell & Jubb, 1977).

### *Biological assay for the catabolic factor*

An assay was developed, suitable for monitoring column fractions, in which the release of chondroitin sulphate from living cartilage in organ culture was measured; bovine nasal septum was used so that large numbers (150) of replicate assays could be done on cartilage from one animal. The cartilage was obtained within 2h of the pig's slaughter, cut into discs (0.5cm diam. × 0.1cm thick), and each was explanted on a Millipore filter supported on a steel ring in a 3cm plastic Petri dish. Each disc was incubated at 37°C in $CO_2$/air (1:19) in 1.5ml of DME medium containing 5% (v/v) heat-inactivated normal sheep serum. The medium was changed after 4 days and the culture terminated after a further 4 days. Test samples (up to 200μl) were included in the culture medium. Each sample was assayed in quintuplicate, and a negative (no test sample) and a positive (known active sample) control was made for each nasal septum. In initial experiments to show the catabolic activity of synovial medium on cartilage, medium from the synovial cultures was mixed with an equal volume of fresh DME medium, and heat-inactivated normal sheep serum was added to 5% (v/v) concentration. The medium from the synovial cultures was not depleted of glucose or amino acids. After culture, chondroitin sulphate was measured in a papain digest of the cartilage and the 4- and 8-day culture medium (Dingle *et al.*, 1973). Initially the Alcian Blue assay was used, but subsequently, for large numbers of samples, a dimethyl Methylene Blue assay (Humbel & Etringer, 1974) was used. It gave results within 5% of those obtained by the Alcian Blue assay and was not affected by culture medium under the conditions used. 1,9-Dimethyl Methylene Blue (16mg) was dissolved by stirring at room temperature for 1h in 100ml of 5% (v/v) ethanol in citrate buffer [0.1M-citric acid/0.2M-$Na_2HPO_4,12H_2O$ (7:3, v/v), pH3.5]; for use, the dye solution was diluted 1:10 with citrate buffer. Samples were added to 5ml of diluted dye solution

and the $\Delta A_{535}$ was measured immediately. The assay was standardized with shark chondroitin sulphate (5–30 $\mu$g). Results were expressed as the percentage of total chondroitin sulphate released, that is:

$$\frac{\text{Amount } (\mu g) \text{ of chondroitin sulphate in medium} \times 100}{\text{Amount } (\mu g) \text{ of chondroitin sulphate in medium + tissue}}$$

*Histology*

Cartilage for histological examination was processed as described by Fell & Jubb (1977).

*$(NH_4)_2SO_4$ fractionation of synovial medium*

Solid $(NH_4)_2SO_4$ was added gradually to solutions to the required saturation at 4°C with stirring. After being left for 1 h, solutions were centrifuged at 10000 rev./min (12000*g*) for 15 min. For the $(NH_4)_2SO_4$-fractionation experiments, precipitates were redissolved in the original volume of DME medium and, together with the final supernatant, were dialysed exhaustively against DME medium. For large-scale preparation of the catabolic factor, the protein precipitating between 60 and 95% saturation was redissolved in phosphate-buffered saline (1.1 mM-$KH_2PO_4$/8.1 mM-$Na_2HPO_4$/137 mM-NaCl/2.5 mM-KCl, pH 7.4) in one-hundredth of the original volume and dialysed exhaustively against phosphate-buffered saline. Dialysis tubing was boiled in 0.1 M-$Na_2CO_3$ and washed in distilled water before use. Protein was measured with the Folin–Ciocalteu reagent (Lowry *et al.*, 1951), with bovine serum albumin as a standard.

*Affinity chromatography and enzymic digestion*

Chick ovoinhibitor was covalently coupled to Sepharose 4B that had been activated with CNBr (Porath *et al.*, 1973). Coupling was carried out at pH 8.5. in 0.1 M-$NaHCO_3$, and the mixture contained 5 mg of ovoinhibitor/ml of gel. The gel bound 1 mg of trypsin/ml.

Enzymic digestions of the catabolic factor were carried out as follows. Samples (5 mg) of 60–95%-$(NH_4)_2SO_4$ precipitate in phosphate-buffered saline were digested for 24 h at 37°C with 100 $\mu$g of bovine trypsin, chymotrypsin or porcine pancreatic elastase. A control sample containing no proteinase was also incubated. After digestion, each sample (including the control) was passed through two 3 ml columns of ovoinhibitor–Sepharose, with phosphate-buffered saline as the column buffer. After the second column the efficiency of removal of proteinase was measured by assay with azocasein substrate (Charney & Tomarelli, 1947). Removal was complete for chymotrypsin and elastase, and better than 99% for trypsin. Protein (160 $\mu$g of each sample) was added to the cartilage assay. The serum in the culture medium was sufficient to inhibit the trace of trypsin present.

## Results

*Effect of synovial medium on cartilage*

Cultivation of porcine articular cartilage in the presence of used medium from cultures of porcine synovium produced degradation of cartilage matrix similar to that seen when the tissues were cultured in the same dish but not in contact (Fell & Jubb, 1977). The used synovial medium also caused release of chondroitin sulphate when assayed on bovine nasal cartilage. The highest activity (75% release, S.E.M. 4%) was found in medium from synovium cultured for 4 days. Dead synovium (frozen and thawed) produced no catabolic activity.

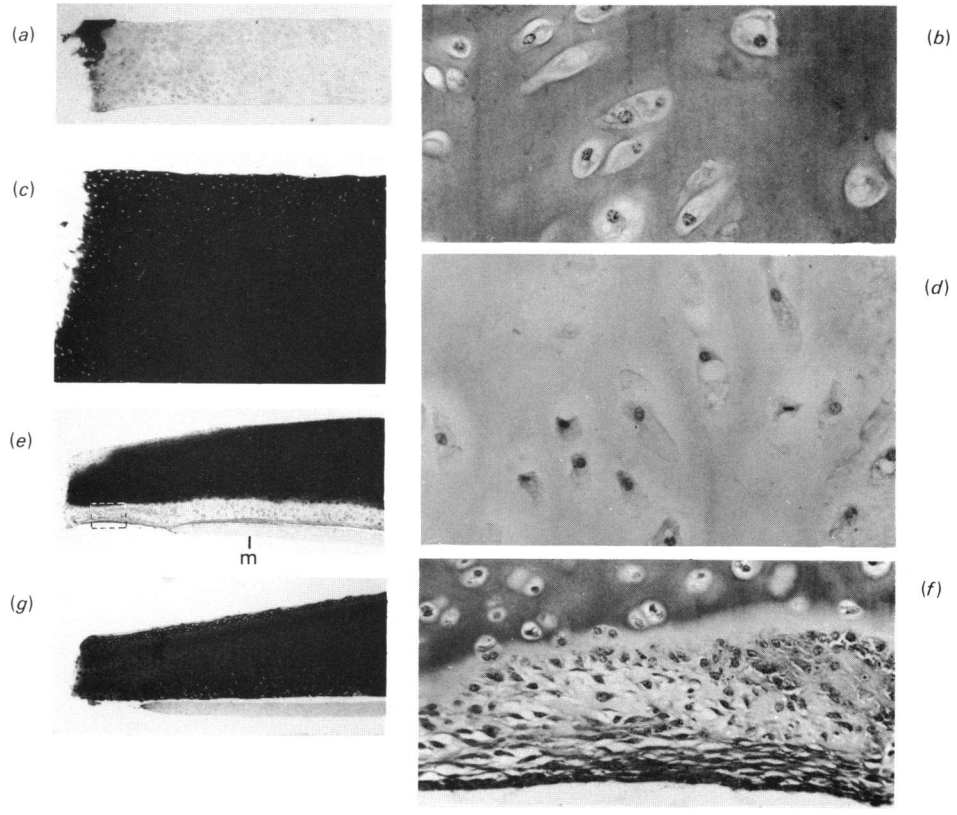
*$(NH_4)_2SO_4$ fractionation of the catabolic factor*

Initial experiments showed that the catabolic factor did not pass through dialysis tubing and that it could be concentrated over an ultrafiltration membrane of 10000-mol.wt. cut-off. Medium concentrated 40-fold was fractionated with $(NH_4)_2SO_4$ by serial additions to 20, 40, 60, 80 and 100% saturation. Table 1(*a*) shows the catabolic activity of the fractions measured in the bovine-nasal-cartilage assay (note that twice as much protein was assayed of the 20, 40 and 60% precipitates as of the 80 and 100% precipitates). Little significant activity was found in the precipitates up to 60% saturation, the major part of the active material precipitated between 60 and 100% saturation. The 100% supernatant was inactive. To fractionate the catabolic factor from unconcentrated synovial culture medium, a 60–95% $(NH_4)_2SO_4$ fraction was made. Fig. 1 shows a dose–response curve obtained by adding increasing amounts of the fraction to the bovine $(NH_4)_2SO_4$-nasal-cartilage assay. The release of chondroitin sulphate increased in an approximately linear fashion as more protein was added. Plates 1(*a*) and (*b*) show the histological appearance of a piece of bovine nasal cartilage corresponding to the highest point in Fig. 1. Plates 1(*e*) and 1(*f*) show the effect of the $(NH_4)_2SO_4$ fraction on porcine articular cartilage. The changes produced are essentially the same as those caused by the unfractionated synovial culture medium.

The $(NH_4)_2SO_4$ fraction had no effect on dead cartilage (Table 1*b*). This result, together with the fact that the proteinases known to be present in the synovial medium, namely collagenase and another metalloproteinase, precipitate at 30–60% $(NH_4)_2SO_4$ saturation (Cawston & Tyler, 1979), show that the effect is not due to proteinases in the synovial medium.

*Lability of the catabolic factor*

The catabolic activity was destroyed by heating the $(NH_4)_2SO_4$ fraction at 70 or 80°C for 10 min (Table 1*b*). The activity was also destroyed by digestion of the fraction with pig pancreatic elastase




## EXPLANATION OF PLATE 1

*Effect of porcine synovial factor on bovine nasal and pig articular cartilage*

(*a*) Vertical section of explant of bovine nasal cartilage after 8 days' culture in the presence of the 60–95%-$(NH_4)_2SO_4$ fraction of synovial medium. Metachromasia are absent, except in one corner where the cells are dead. Staining was with Toluidine Blue; magnification ×20. (*b*) Horizontal section of a similar explant showing viable chondrocytes. Staining was with Celestine Blue, Carazzi's haematoxylin and van Gieson's stain; magnification ×250. (*c*) As (*a*), but cultured in control medium. There was no loss of metachromasia. Staining was with Toluidine Blue; magnification ×20. (*d*) As (*b*), but culture was in control medium. Staining was as in (*b*) and shows viable chondrocytes; magnification ×250. (*e*) Porcine articular cartilage after 10 days' culture in the presence of the 60–95%-$(NH_4)_2SO_4$ fraction of synovial medium. The peripheral loss of metachromasia should be noted. Staining was with Toluidine Blue ×20; 'm' indicates the Millipore filter. (*f*) Another section of the same explant [approximately within the area marked by the broken line in (*e*)] showing early breakdown of collagen. The chondrocytes are undergoing a fibroblastic transformation. Staining was with Celestine Blue, Carazzi's haematoxylin, and van Gieson's stain; magnification ×188. (*g*) Paired explant of (*a*) grown in control medium. There was no loss of metachromasia. Staining was with Toluidine Blue; magnification ×20.

Table 1. *Results obtained by the bovine-nasal-cartilage assay*

Each part of the Table, (*a*), (*b*) or (*c*), shows results obtained with cartilage from a different bovine nasal septum. The control release of chondroitin sulphate is shown for each septum, and percentages are mean results from quintuplicate assays. In parts (*b*) and (*c*), the '$(NH_4)_2SO_4$ fraction' is the protein precipitating between 60 and 95% saturation. See the text for details. Student's *t* test was used to assess the significance of differences between groups. *Significantly different from control, $P = 0.01$; **significantly different from control, $P = 0.05$; ***not significantly different from control; ****results not significantly different from each other but are from control, $P = 0.01$.

| | State of cartilage | Test sample | Sample protein (μg/culture) | Total chondroitin sulphate released ± S.E.M. (%) |
|---|---|---|---|---|
| (*a*) | Live | 40×-Concentrated synovial medium | 100 | 85.8 ± 11.3* |
| | | | 50 | 67.0 ± 3.3* |
| | | $(NH_4)_2SO_4$ precipitate, 0–20% | 400 | 39.6 ± 5.1** |
| | | 20–40% | 400 | 34.5 ± 2.1** |
| | | 40–60% | 400 | 28.4 ± 2.7*** |
| | | 60–80% | 200 | 88.7 ± 5.6* |
| | | 80–100% | 200 | 58.8 ± 7.1* |
| | | 100% $(NH_4)_2SO_4$ supernatant | 200 | 22.4 ± 4.2*** |
| | | None | 0 | 26.0 ± 2.0 |
| (*b*) | Live | None | 0 | 21.4 ± 1.7 |
| | | $(NH_4)_2SO_4$ fraction | 50 | 66.2 ± 4.4 |
| | Dead | None | 0 | 20.0 ± 1.9 |
| | | $(NH_4)_2SO_4$ fraction | 50 | 20.8 ± 2.6 |
| | Live | $(NH_4)_2SO_4$ fraction 70°C, 10min | 50 | 21.8 ± 2.4 |
| | | 80°C, 10min | 50 | 24.0 ± 2.5 |
| (*c*) | Live | None | 0 | 29.6 ± 2.2 |
| | | $(NH_4)_2SO_4$ fraction | | |
| | | Undigested | 150 | 67.8 ± 9.3**** |
| | | +Trypsin | 150 | 57.0 ± 6.8**** |
| | | +Chymotrypsin | 150 | 41.2 ± 6.1*** |
| | | +Elastase | 150 | 34.0 ± 4.4*** |

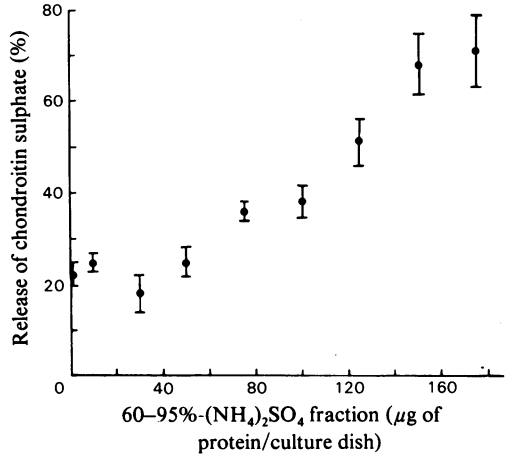



Fig. 1. *Dose–response curve for bovine-nasal-cartilage assay*

Increasing amounts of the 60–95% $(NH_4)_2SO_4$ fraction of synovial medium were added to the cartilage assay. Points represent mean results from quintuplicate assays, and the vertical bars represent the S.E.M. The indicated amount of protein was present in the original medium and at the change at 4 days.

or bovine chymotrypsin, but not by bovine trypsin (Table 1*c*), suggesting that the activity is due to a protein; this is consistent with its thermolability and with the fact that it does not pass a 10000-mol.wt.-cut-off ultrafiltration membrane. The failure of trypsin to destroy the catabolic activity may be due to its having a different and more limited specificity for peptide bonds than has chymotrypsin or pancreatic elastase. Trypsin catalyses hydrolysis of peptide bonds adjacent to the carbonyl group of the basic L-amino acids arginine and lysine. Chymotrypsin cleaves bonds whose carbonyl function is provided by aromatic L-amino acid residues (tyrosine, tryptophan and phenylalanine) and will cleave more slowly adjacent to other large hydrophobic residues (histidine, leucine and methionine). Pancreatic elastase has a broad specificity with a preference for small hydrophobic side chains. Preliminary gel-filtration chromatography on AcA54 Ultrogel has shown that the catabolic activity is associated with material of about 20000 mol.wt.

## Discussion

The breakdown of cartilage matrix caused by porcine synovial culture medium is due to a protein,

tentatively named 'catabolin' (Dingle, 1979). Catabolin is thought to act directly on living chondrocytes, stimulating them to degrade matrix macromolecules. We do not yet know whether such catabolic proteins are concerned in the regulation of chondrocyte activity *in vivo*, but in diseases such as rheumatoid arthritis and osteoarthritis, where articular cartilage is destroyed, it can be postulated that such substances could substantially influence chondrocyte activity and hence the degree of articular damage.

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