Biochem. J. (1980) **190**, 431–438
Printed in Great Britain

The site of cartilage matrix degradation

John T. DINGLE and Timothy T. DINGLE*
Tissue Physiology Department, Strangeways Research Laboratory, Worts' Causeway, Cambridge CB1 4RN, U.K.

(*Received 30 January 1980*)

1. A 'cavity' organ culture method was developed, which allowed the insertion within bovine nasal cartilage of 10μl of ^{35}S-labelled proteoglycan aggregate. 2. Stimulation of the tissue with retinol or catabolin led to degradation of both the cartilage matrix and the proteoglycan within the cavity. 3. Proteoglycan aggregate trapped in polyacrylamide beads was not broken down in the cavities of living cartilage, except under autolytic conditions. 4. It is suggested that degradation of proteoglycan aggregate by chondrocytes occurs in a pericellular region.

The crippling nature of arthritic disease is largely due to the breakdown of the matrix of articular cartilage. It has become widely held that this degradation is caused by the diffusion of proteolytic enzymes, principally the neutral metalloproteinases, into the articular cartilage from adjacent synovial tissue or inflammatory cells (Kobayashi & Ziff, 1975; Harris & Cartwright, 1977). However, the recent finding (Dingle *et al.*, 1979) that synovial tissue can initiate drastic cartilage resorption by the release of non-enzymic factors (catabolins), which act directly on living chondrocytes causing these cells to degrade the surrounding matrix, has re-opened the question of the site of matrix catabolism within resorbing cartilage.

The experiments reported in this paper make use of a new technique of cavity organ culture (Dingle, 1979). In this method a sealed cavity is formed within a block of bovine nasal cartilage. The cavity is filled with characterized and radiolabelled matrix polymers and the block incubated under controlled catabolic conditions. Experiments made by this technique lead us to suggest that the extracellular catabolism of matrix macromolecules by soluble tissue proteinases is unlikely. A pericellular site for proteinase action is proposed.

Materials and methods

Cavity organ culture

This technique has been described briefly (Dingle, 1979), and is illustrated diagramatically in Fig. 1.

Abbreviations used: PGA, proteoglycan aggregate; [^{35}S]PGA, ^{35}S-labelled proteoglycan aggregate.

* Present address: Department of Biological Sciences, University of East Anglia, Norwich, U.K.

Bovine nasal cartilage from young adult animals was obtained within 2h of the cow's slaughter, the connective tissue was removed and the cartilage was cut into blocks 13mm × 4mm × 4mm. A small hole was drilled into each block, which was then closed at the end with a stainless steel screw. Polymeric material did not leak from these cavities, but did from similar cavities made in young adult ovine and rabbit cartilage, precluding the use of these tissues. The volume of the enclosed cavity was approx. 10μl. The cartilage blocks were cultured under conventional organ culture conditions, similar to those used for bovine nasal cartilage discs (Dingle *et al.*, 1979). The use of a stainless steel template for cutting and drilling the cartilage allowed up to 50 uniform blocks to be prepared for an experiment. In most experiments the tissue was cultured for 6 days, the medium being changed on day 3. In some experiments the cavities were filled at day 5 and incubation was continued for up to 24h subsequently. The medium used in all experiments was BGJ_5, containing 5% (v/v) heat-inactivated normal sheep serum (Dingle *et al.*, 1977). The cavity organ cultures were cultivated in medium alone or in medium containing an activating agent, either retinol (3μg/ml) or an $(NH_4)_2SO_4$ fraction (60–90% satd.) of catabolin (20μg of protein/ml) (Dingle *et al.*, 1979). In most experiments 10μl of a solution (1mg/ml) of [^{35}S]PGA in 0.9% NaCl was added to the cavities. In some experiments, a suspension in 0.9% NaCl of polyacrylamide beads labelled with ^{14}C and containing [^{35}S]PGA was added. Material was recovered from the cavities after various times in culture by centrifugation for 10min at 1000*g*, by using a shoulder tube formed from part of a 1ml plastic syringe barrel, a procedure developed by T.

0306-3283/80/080431-08$01.50/1

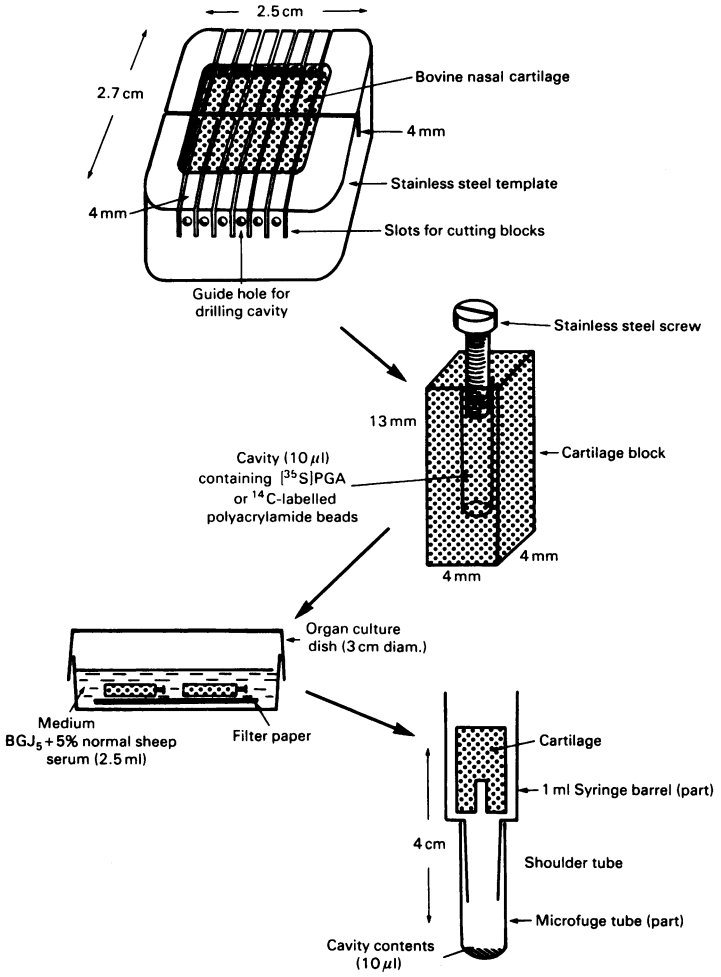

Fig. 1. *Cartilage cavity organ culture*

The diagrams are representative of the procedures set out in the Materials and methods section for preparing the cartilage, culturing and harvesting the cavity contents. The diagrams are not to scale. All procedures were carried out under aseptic conditions. The cartilage blocks were cut and drilled in the template. After the cavities were filled with 10 μl of substrate they were closed with a stainless steel screw and the cartilage was incubated at 37°C in a culture dish in medium BGJ_5, containing 5% (v/v) heat-inactivated normal sheep serum. The gas atmosphere was $O_2/CO_2/N_2$ (4:1:15, by vol.). The cavity contents were recovered by centrifuge in a shoulder tube formed from a 1 ml plastic syringe barrel and a Microfuge (Beckman Instruments) tube.

T. Dingle (Dingle, 1979) (Fig. 1). A recovery of 90–95% was obtained, based on scintillation counting of either [^{14}C]polyacrylamide or [^{35}S]PGA, and also on assays of chondroitin sulphate. The effect of the stimulating agents on the degradation of the cartilage matrix of the organ culture was measured by assay of the release of chondroitin sulphate, results being expressed as a percentage of the initial content of the matrix, as described by Dingle *et al.* (1979). The tissue content of glycosaminoglycan, and the released glycosaminoglycan, was measured by the dimethyl Methylene Blue dye method of Humbel & Etringer (1974) (Dingle *et al.*, 1979) using a 0.1 M-formate buffer, pH 3.5, instead of a citrate buffer, a modification introduced by C. A. Sayers & A. J. Barrett (personal communication). All culture experiments were set up in quintuplicate, with two blocks of cartilage in each of the five culture dishes.

Preparation and assay of [^{35}S]*PGA*

Bovine nasal cartilage was labelled with $Na_2{}^{35}SO_4$ as described by Dingle *et al.* (1977). PGA was prepared from this cartilage by the method of

Hascall & Heinegard (1974) and final purification was by chromatography on preparative Sepharose 4B columns equilibrated and eluted as described below. The excluded material was used in the cavity experiments. After culture the ^{35}S-labelled proteoglycan recovered from the cavities was analysed on small Sepharose 4B columns (0.6cm × 16cm; 4.5ml) equilibrated with 0.2M-acetate buffer, pH5.5. Usually about 5000c.p.m. of ^{35}S were placed on the column in 20μl (from the two blocks in each culture dish), and 60 fractions (each 120μl) were collected. Results are expressed as the ratio (× 100) of the counts in each fraction (F) to the total counts (T) [i.e. $(F \times 100)/T$]. In some experiments the diffusion of ^{35}S-labelled proteoglycan into the culture medium was measured after 10-fold concentration over Carbowax (R. A. Lamb, London, U.K.).

Preparation of cross-linked polyacrylamide beads

The method was essentially that described by Dingle *et al.* (1977) except that *NN'*-[^{14}C]methylenebisacrylamide was used. This was a gift from Dr. G. Knight of this laboratory, who prepared it from [^{14}C]paraformaldehyde by the method of Feuer & Lynch (1953).

The incorporation of [^{35}S]PGA into the ^{14}C-labelled beads was carried out as previously described (Dingle *et al.*, 1977). Digestion of the beads for 18h at 60°C with papain (0.1mg) in the presence of 2.0mM-EDTA, 5.0mM-cysteine and 0.1M-phosphate buffer, pH6.5, followed by two washes with 0.9% NaCl removed all the [^{35}S]PGA. Digestion and wash volumes were 500μl. The washed beads were counted for ^{14}C and the papain digest and washings were counted for ^{35}S in a liquid-scintillation counter (Packard Tri-Carb) as previously described (Dingle *et al.*, 1977).

The above method was used to determine the residual proteoglycan in the beads after incubation in the cavity. The following calculation allowed the determination of the percentage breakdown of proteoglycan in the beads. Digestion under standard conditions of the unincubated beads gave a ratio of initial papain-soluble counts (S_0) to insoluble counts (I_0), $S_0/I_0 = R$. To calculate percentage digestion after incubation:

1. Papain-insoluble counts (I) × ratio (R) = initial PGA content of the beads in the cavity.
2. S = the papain-soluble counts in the beads after culture.
3. Percentage digestion $= \dfrac{(IR-S)}{IR} \times 100.$

Measurements on recovery from eight cavities by this method gave a mean ratio R of 0.23 with a S.E.M. of 0.01. Approx. 1500c.p.m. of [^{35}S]PGA and 6000c.p.m. of [^{14}C]polyacrylamide (papain-insoluble) counts were placed in each cavity.

Results

Validation of the cavity organ culture system

The release of proteoglycan from stimulated and unstimulated bovine nasal cartilage in organ culture is shown in Fig. 2. Dead (frozen and thawed twice) or unstimulated cartilage released less than 20% of the tissue proteoglycan content during 9 days in culture. Living cartilage stimulated with retinol released approx. 60%, whereas stimulation with catabolin resulted in a substantial release of proteoglycan by 6 days and nearly complete loss by 9 days.

The release of matrix proteoglycan from bovine nasal cartilage showed a delayed response to retinol stimulation (Fig. 2). This delay was reflected in experiments in which PGA was incubated within the cavities of retinol-stimulated cartilage for three periods; there was little change in molecular size in

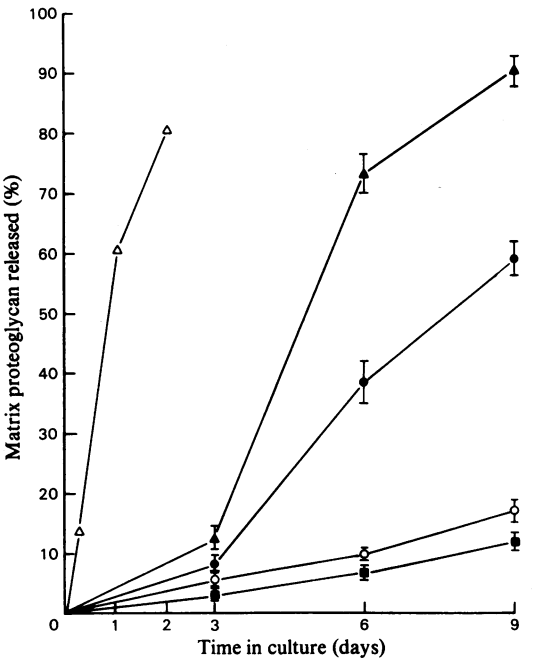

Fig. 2. *Release of cartilage matrix proteoglycan*
Blocks of bovine nasal cartilage were cultured for 9 days; the control medium was changed every 3 days and the released polysaccharide was measured. The results are expressed as a percentage of the total proteoglycan. The S.E.M. of quintuplicate cultures is shown. ■, Dead tissue; ○, unstimulated cartilage; ●, cartilage stimulated with 3μg of retinol/ml; ▲, cartilage stimulated with 20μg of catabolin/ml. Dead cartilage blocks were also incubated at 37°C under autolysing conditions at pH5.5, in 0.1M-acetate buffer (△). Results are the means of triplicates.

the first 0–3 day period, whereas from 3 to 6 days breakdown was clearly evident, and was sufficient in the final period to cause loss from the cavity, probably by diffusion of the low-molecular-weight products through the cartilage to the external medium (Fig. 3).

The breakdown of the PGA in these and subsequent experiments was not due to endogenous enzymes that had remained throughout the purification procedures, since incubation *in vitro* either at pH 7 (Fig. 4) or at pH 5.5 did not result in appreciable changes in molecular size. It is also shown in Fig. 4 that incubation of [^{35}S]PGA for 16 h at pH 7 in a cavity made in dead tissue did not result in any appreciable change in molecular size, a result consistent with the small degree of matrix breakdown seen during incubation of dead cartilage (Fig. 2). However, autolysis of cartilage in acetate buffer at pH 5.5, which led to rapid breakdown of matrix (Fig. 2), also resulted in a very substantial change in the molecular size of the [^{35}S]PGA within the cavities (Fig. 4).

A drawback of the retinol-stimulated-cartilage experiments was the necessity to incubate the PGA in the cavities for approx. 3 days (i.e. from day 6 to day 9) to obtain a substantial effect on molecular size. For this reason an $(NH_4)_2SO_4$ preparation of catabolin which gave very rapid and substantial degradation (Fig. 2 and Fig. 6) was used in subsequent experiments. When this stimulation was applied for 5 days before the [^{35}S]PGA was placed in the cavity, a retardation of the ^{35}S-labelled

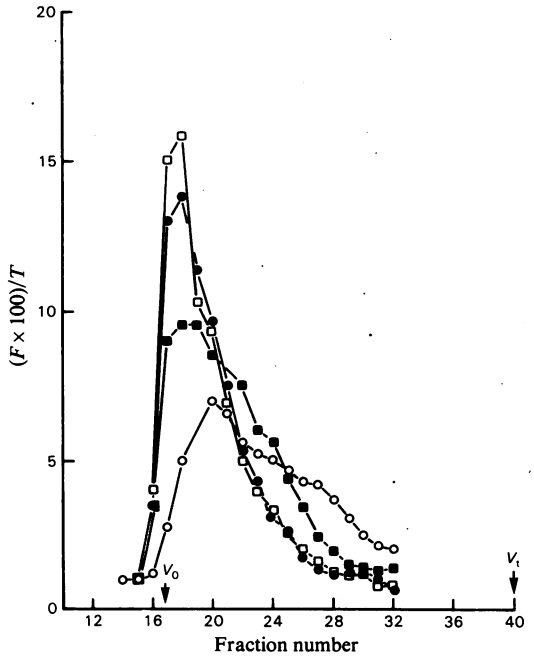

Fig. 3. *Degradation of* [^{35}S]*PGA in cavities of retinol-stimulated cartilage*

Cavity organ cultures containing [^{35}S]PGA were incubated for days 0–3, days 3–6 and days 6–9 in the presence of retinol (3 μg/ml). The cavity contents were chromatographed on Sepharose 4B columns. The results are expressed as the ratio of c.p.m./fraction (F) (× 100) to the total c.p.m. (T) [i.e. $(F \times 100)/T$] and are the means of quintuplicate cultures, each analysed separately. □, Unincubated [^{35}S]PGA; ●, [^{35}S]PGA recovered after days 0–3 in cavity; ■, [^{35}S]PGA recovered after days 3–6 in cavity; ○, [^{35}S]PGA recovered after days 6–9 in cavity. V_0, void volume; V_t, total volume of the column.

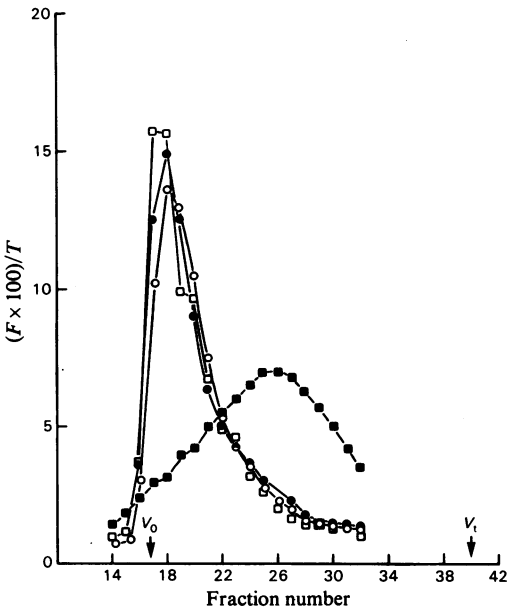

Fig. 4. *Degradation of* [^{35}S]*PGA in cavities made in dead cartilage*

[^{35}S]PGA was incubated at 37°C in 0.1 M-phosphate buffer, pH 7, for 16 h. Cavities were made in dead cartilage and, after filling with [^{35}S]PGA, were incubated in culture medium (pH 7.2) or under autolysing conditions in 0.1 M-acetate buffer, pH 5.5. The results of chromatography on Sepharose 4B are expressed as described in the legend to Fig. 3 and are the means of separate estimations on quintuplicate cultures. Unincubated [^{35}S]PGA is also shown. ●, [^{35}S]PGA incubated in tubes at pH 7 for 16 h; ○, [^{35}S]PGA incubated in cavities of dead cartilage at pH 7 for 16 h; ■, [^{35}S]PGA incubated in cavities of dead cartilage at pH 5.5 for 16 h; □, unincubated [^{35}S]PGA. V_0, void volume; V_t, total volume of the column.

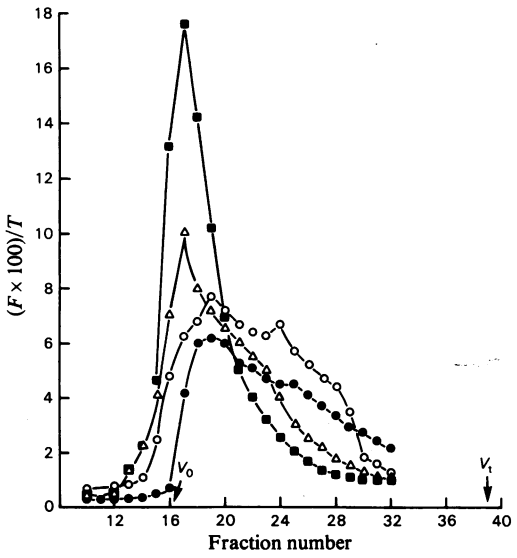

Fig. 5. *Degradation of [^{35}S]PGA in cavities of catabolism-stimulated cartilage*

After 5 days in culture in the presence of 20μg of catabolin/ml the cavities in bovine nasal cartilage blocks were filled with 10μl of [^{35}S]PGA (1mg/1ml). Culture was continued for 3, 6 or 12h, and at these times the cavity contents were recovered by centrifugation. The [^{35}S]PGA was chromatographed on columns of Sepharose 4B, and the results are expressed as described in the legend to Fig. 3. The results are the means of separate estimations on quintuplicate cultures. ■, Unincubated [^{35}S]PGA; △, 3h incubation in cavities; ○, 6h incubation in cavities; ●, 12h incubation in cavities, V_0, void volume; V_t, total volume of the column.

proteoglycan on Sepharose 4B could be demonstrated within 3h (Fig. 5). By 12h, a substantial diminution of molecular size was apparent. Prolonging the experiment further led to loss of degraded material from the cavity by diffusion through the tissue into the culture medium (Fig. 6). Incubation of [^{35}S]PGA within cavities of unstimulated cartilage that had been cultured for 5 days in control media showed much less breakdown of material, although after 12h of incubation a clearly defined shoulder on the aggregate peak was demonstrated by chromatography on Sepharose 4B (Fig. 7). There was very little loss of [^{35}S]PGA from cavities in unstimulated cartilage.

Experiments with polyacrylamide beads in cavities

The quantification of the degradation of bead-associated PGA within the cavity suffered from two difficulties: (1) it was impossible to place exactly the same quantity of beads in each cavity, and (2) low recovery of the released radioactivity due to diffusion artifacts was a possibility. The double-labelling technique overcame the errors implicit in filling the cavities with a slurry of beads, since an accurate estimate of bead quantity was given by the papain-insoluble counts. Recovery of released ^{35}S radioactivity associated with degraded material was found to be inaccurate when extensive degradation occurred, due to the diffusion of low-molecular-weight material into the cartilage and eventually out into the medium. Hence the assay of residual counts in the beads was used as a measure of degradation. This method required an accurate estimate of the quantity of [^{35}S]PGA initially placed in the cavity. Double labelling of the beads allowed this.

The results in Table 1 demonstrate the release of proteoglycan from beads in blank test tube incubations and in cavities of both unstimulated and catabolin-stimulated cartilage. There is no significant release of bead-bound [^{35}S]PGA after incubation in cavities in either stimulated or unstimulated cartilage, over that of the blank incubations. As a control the beads were placed in cavities in dead (frozen and thawed) cartilage under autolytic conditions (pH5.5) for similar lengths of time. In these circumstances there was rapid and substantial release of ^{35}S-labelled proteoglycan from the beads (87% in 24h).

Comparison of sensitivity to enzyme degradation of PGA in beads with that of soluble PGA

It seemed possible that the lack of breakdown seen with proteoglycan contained in beads was due to enzyme levels being below the level of sensitivity of the method. Change in molecular size of the soluble aggregate might have been a more sensitive indicator of enzymic activity. The change in retardation on Sepharose 4B is not a quantitative method, but the limit of observable effect can be determined. For trypsin acting on soluble PGA (1mg/ml) this was established as 5–50ng of trypsin/ml for a 6h incubation at 37°C in 0.2M-Tris/HCl, pH7.6. Incubating bead-bound PGA under the same conditions yielded a limit of sensitivity of 5ng of trypsin/ml. From these experiments it seems unlikely that the difference in sensitivity of assay is the reason for the observed difference in effect on proteoglycan within the polyacrylamide beads as compared with soluble material.

Discussion

The cavity organ culture technique allows the insertion of radiolabelled macromolecules into an environment that should approximate to that within living cartilage. If this has been achieved, the pattern of degradation of proteoglycan inside the cavities

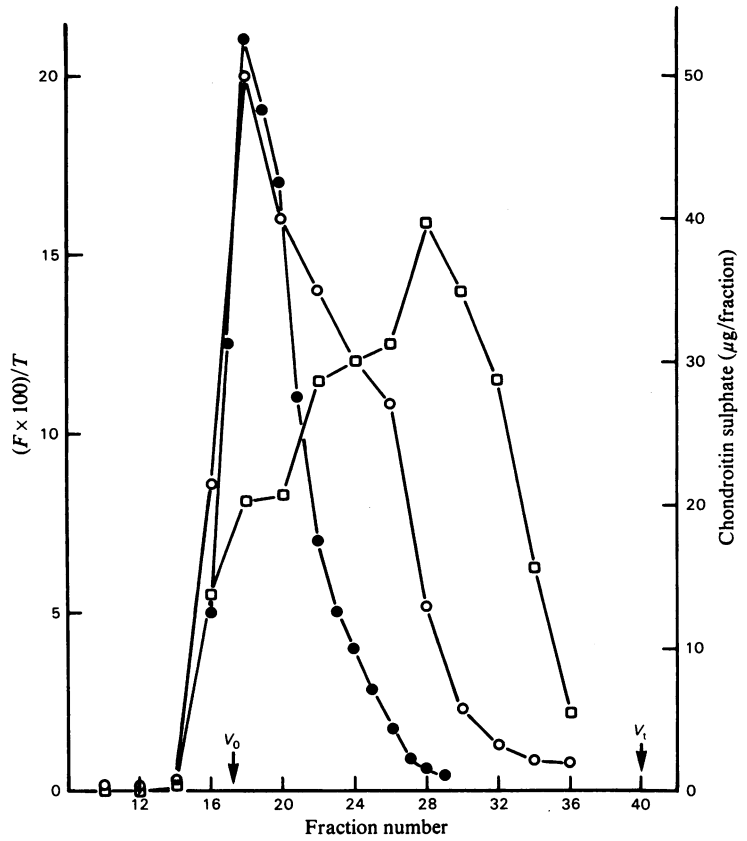

Fig. 6. *Release of proteoglycan into the culture medium*

The conditions of culture are as described in the legend to Fig. 5 except that the culture was continued for 24 h after filling the cavities. The medium from the culture was concentrated 10-fold over Carbowax and chromatographed on Sepharose 4B. The results are expressed as described in the legend to Fig. 3 for the [^{35}S]PGA release from the cavity and as μg of chondroitin sulphate/fraction for the matrix proteoglycan released into the medium. [^{35}S]PGA was also added to the medium and concentrated in the same manner to determine if any change in molecular size occurred during the concentration procedures. ●, [^{35}S]PGA concentrated from the medium; □, [^{35}S]PGA released into the medium from the cavity; ○, proteoglycan released from cartilage matrix. V_0, void volume; V_t, total volume of the column.

should be similar to that of the constituent proteoglycan of the intact cartilage matrix.

Dingle (1979) demonstrated that ^{35}S-labelled cartilage proteoglycan subunit was appreciably degraded when incubated from day 3 to day 6 within a cavity formed in bovine nasal cartilage in the presence of retinol (3 μg/ml). In the present study it was thought preferable to use cartilage proteoglycan aggregate (PGA) since the indigenous matrix proteoglycan occurs in this form (Hardingham & Muir, 1974).

Morrison (1970) studied the behaviour on gel chromatography of matrix proteoglycan released from embryonic chick cartilage in organ culture after stimulation with retinol. He demonstrated changes in molecular size that were consistent with a proteolytic attack on the protein core of the macromolecule. In the present experiments the retardations on Sepharose 4B of matrix proteoglycan released into the culture medium and of digestion products from PGA incubated within cartilage cavities appeared to be similar to those shown by Morrison (1970).

The lack of effect on the PGA contained within cavities of dead tissue at neutral pH is consistent with the stability of the proteoglycan of the cartilage matrix itself to incubation under these conditions. The stimulation of breakdown of PGA within cavities in living tissue occurred with the same agents and with a similar time relationship to that

seen in the degradation of the cartilage matrix surrounding the cavities. These findings are taken as evidence that similar enzymic mechanisms are responsible for the degradation of the soluble PGA in the cavity and for the digestion of the matrix of the living tissue. In the light of these findings the cavity organ culture technique should prove useful in determining the nature of enzyme attack on matrix polymers, since the serious difficulties imposed on analytical studies by matrix heterogeneity can be largely overcome by using characterized radiolabelled materials in the cavity. The method also distinguishes between effects on synthesis and degradation in living cartilage.

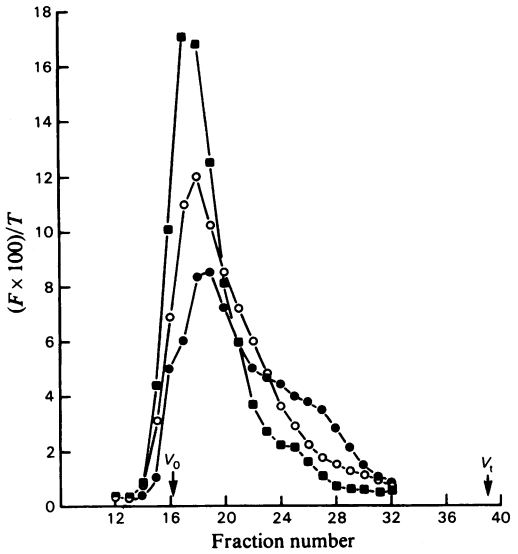

Fig. 7. *Degradation of [^{35}S]PGA in cavities of unstimulated cartilage*

The conditions of cultures and assay are as described in the legend to Fig. 5, except that no catabolin was present. ■, Unincubated [^{35}S]PGA; ○, [^{35}S]PGA incubated for 6h in cavities; ●, [^{35}S]PGA incubated for 12h in cavities. V_0, void volume; V_t, total volume of the column.

The very rapid breakdown of soluble PGA in cavities after stimulation by catabolin is absent when the aggregate is bound in polyacrylamide beads within the cavities. It should be pointed out that Dingle *et al.* (1977) were able to demonstrate that trapping proteoglycan within beads did not hinder the action of a wide variety of soluble proteinases. Furthermore, the rapid hydrolysis of the PGA in beads within the cavities under autolytic conditions demonstrates the availability of the bead-bound substrate to cartilage enzymes that are rendered soluble by freezing and thawing; these would include the lysosomal proteinases. These results are compatible with the hydrolysis of soluble PGA in the cavities of living cartilage being due to proteolytic enzymes that are not freely diffusible into the extracellular environment in an active state. When the substrate is hindered, as it is in the polyacrylamide beads, which cannot penetrate the matrix surrounding the cells, active enzyme cannot reach the substrate molecules. However, the soluble PGA is able to diffuse to sites of enzyme activity that may be adjacent to the cavity. As an alternative explanation the possibility exists that cartilage matrix catabolism is due to an as yet unidentified soluble proteinase that is unable to penetrate into the beads, perhaps because of a very high molecular weight.

Histological observations on catabolin-stimulated degradation of bovine nasal cartilage in organ culture (Dingle *et al.*, 1979) showed total loss of metachromasia except in a small focal area where the cells were dead. This is thought to support the concept of localized enzyme action. Since apparently normal chondrocytes are present up to the edge of the cavity, it seems likely that the proteinases

Table 1. *Release of [^{35}S]PGA from beads in cartilage cavities*

^{14}C-Labelled polyacrylamide beads containing [^{35}S]PGA were suspended in 0.9% NaCl and incubated in cavities in control (unstimulated) or catabolin-stimulated cartilage. The cartilage was cultured for 5 days before the addition of the beads and the culture was continued for up to a further 24h. The recovery and estimation of bound [^{35}S]PGA in the beads is described in the text. Beads were also incubated at 37°C in cavities in dead cartilage under autolysing conditions (0.1M-acetate buffer, pH 5.5) and, as a control *in vitro*, in 50μl of 0.9% NaCl buffered with 0.1M-phosphate, pH 7.2, in 500μl tubes. All estimates were in quintuplicate and the S.E.M. is shown.

	Percentage of [^{35}S]PGA released from:			
Time (h)	Control *in vitro* (no cartilage)	Unstimulated cartilage in culture	Catabolin-stimulated cartilage in culture	Autolysis of dead cartilage
6	5.9 ± 1.7	4.7 ± 0.8	6.1 ± 2.0	31 ± 3
12	11.7 ± 1.8	9.3 ± 1.2	8.9 ± 2.3	49 ± 4
24	18.0 ± 1.3	17.0 ± 1.7	20.6 ± 3.6	87 ± 7

responsible for degradation of the cavity PGA are bound to, or only active within a short distance of, the plasma membrane of these cells.

The above results are in agreement with the result of an earlier, but much cruder, attempt to devise a culture sytem that would limit the accessibility of proteoglycan to non-diffusible enzymes. In this so-called 'hamburger culture' (Dingle, 1976) ^{35}S-labelled proteoglycan subunit was contained in a polyacrylamide disc that was placed in an annulus of rabbit ear cartilage and two further discs of cartilage were placed one on each side. During 3 days in culture the perichondrium of the cartilage grew together, effectively sealing in the polyacrylamide disc. Subsequent stimulation by retinol led to degradation of the cartilage matrix, but had no effect on the proteoglycan contained in the polyacrylamide disc. These experiments led us to suggest that the local degradation of matrix molecules may occur in a functional pericellular microenvironment of a stimulated chondrocyte.

This region may be related to the pericellular microenvironment of chondrocytes postulated from ultrastructural studies by Meachim & Stockwell (1973) and by Dingle (1975). Since the matrix of isolated cartilage may be degraded by the action of stimulated chondrocytes without the aid of extrinsic enzymes, we further suggest, in the light of the recently discovered action of the synovial factor catabolin, that the degradative activity occurring in arthritic cartilage cannot be ascribed entirely to the release of synovial or other extrinsic proteinases. The local action of chondrocyte enzymes may well play a significant role in the disease process.

The authors wish to thank Mrs. Bridget Wright and Mrs. Beryl King for their skilled technical assistance.

References

Dingle, J. T. (1975) *Phil. Trans. R. Soc. London Ser. B* **271**, 315–324

Dingle, J. T. (1976) *Proteolysis Physiol. Regul. Proc. Miami Winter Symp.* **11**, 339–353

Dingle, J. T. (1979) *Ann. Rheum. Dis.* **38**, 201–214

Dingle, J. T., Blow, A. M. J., Barrett, A. J. & Martin, P. E. N. (1977) *Biochem. J.* **167**, 775–785

Dingle, J. T., Saklatvala, J., Hembry, R., Tyler, J., Fell, H. B. & Jubb, R. (1979) *Biochem. J.* **184**, 177–180

Feuer, H. & Lynch, E. (1953) *J. Am. Chem. Soc.* **75**, 5027–5029

Hardingham, T. E. & Muir, H. (1974) *Biochem. J.* **139**, 565–581

Harris, E. D. & Cartwright, E. C. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 249–283, North-Holland, Amsterdam

Hascall, J. C. & Heinegard, D. (1974) *J. Biol. Chem.* **249**, 4232–4241

Humbel, R. & Etringer, D. (1974) *Rev. Roum. Biochim.* **11**, 21–24

Kobayashi, I. & Ziff, M. (1975) *Arthritis Rheum.* **18**, 475–483

Meachim, G. & Stockwell, R. A. (1973) in *Adult Articular Cartilage* (Freeman, M. A. R., ed.), pp. 51–99, Pitman, London

Morrison, R. I. G. (1970) Ph.D. Dissertation, University of Cambridge, Cambridge, U.K.