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A direct simultaneous plate assay of proteoglycan and collagen degradation by cells in culture and its application to synovial cells

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1. A radiochemical plate assay is presented that allows a simultaneous evaluation of the capacity of cells in culture to degrade proteoglycan and collagen. Its principle consists of monitoring the release of soluble radioactive degradation products from Multiwell culture plates coated with dried reconstituted ^{3}H-labelled-proteoglycan/^{14}C-labelled-collagen mixed gels. The plates can also be used for the assay of proteolytic activities within enzyme solutions. 2. When cultured on the plates, rabbit synovial cells degrade collagen and proteoglycan almost simultaneously, owing to the secretion of collagenase and of a proteoglycan-degrading metal-dependent neutral proteinase.

Investigations of the biological mechanisms underlying the degradation of cartilage and other connective tissues require convenient and sensitive assays for the enzymes that are responsible for the lysis of the main structural macromolecules of these tissues, proteoglycan and collagen. The first attack on these insoluble matrices is extracellular, and therefore requires the action of hydrolytic enzymes secreted by effector cells or bound to their external surface. The soluble fragments produced can then diffuse away and/or be further degraded, either extracellularly or intracellularly, after their endocytic uptake in phagolysosomes.

We have developed a radiochemical proteoglycan/collagen plate assay that allows evaluation of the capacity of cells cultured in the plates to degrade these two macromolecules into soluble fragments. Moreover, the plates can be used for the assay of proteoglycan-degrading proteinases or of mixtures of such enzymes and of collagenase in tissue extracts or in enzyme solutions. The principle of the assay consists of monitoring the release of soluble radioactive degradation products from a dish coated with a dried reconstituted ^{3}H-labelled-proteoglycan/^{14}C-labelled-collagen mixed gel. With that assay, we observed that synovial cells are able to degrade both substrates, owing to the release of a proteoglycan-degrading neutral proteinase and of collagenase.

Materials and methods

Materials

Guanidinium chloride was obtained from Baker Chemicals (Deventer, The Netherlands); $NaBH_4$ and aq. 40% (w/v) formaldehyde were from Merck A.G. (Darmstadt, Germany); NaB^3H_4 (20 Ci/mmol) was from CEA (Gif-sur-Yvette, France); crude trypsin ('Bacto Trypsin') was from Difco (Detroit, MI, U.S.A.); twice-crystallized trypsin was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); crude bacterial collagenase (Type I), chromatographically purified collagenase (Type IV), chondroitinase ABC, twice-crystallized papain, bovine testicular hyaluronidase (Type VI), diphenylcarbamoyl chloride-treated trypsin and 4-hydroxymercuribenzenesulphonic acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); human plasmin (15 casein units/mg) was from Kabi (Stockholm, Sweden); Multiwell plates were from Costar (Cambridge, MA, U.S.A.); Sepharose 6B, CL-2B or CL-6B were from Pharmacia AB (Uppsala, Sweden). All the other enzymes, culture media and special chemicals used were from the same suppliers as in our previous work (Vaes, 1972; Hauser & Vaes, 1978).

Cultures of synovial cells

Adherent synovial cells were obtained from either normal or arthritic rabbits (Cooke & Jasin, 1972) by proteolytic dispersion by the method of Dayer *et al.* (1976). The cells were cultured in a water-saturated atmosphere of air/CO_2 (9:1) in Dulbecco's modified Eagle's medium (Smith *et al.*, 1960) supplemented with penicillin (10^5 units/l), streptomycin (100 mg/l) and 10% (v/v) heat-inactivated (30 min at 56°C) foetal calf serum. They were passaged at 1-week intervals. After detachment of the cells with 0.1% crude trypsin in phosphate-buffered saline (Hauser & Vaes, 1978), 1 ml of calf serum/4 mg of

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trypsin used was added to the chilled cell suspension before centrifugation to inhibit trypsin completely and to prevent its interference in the experiments; this was followed by two washings with a large excess of culture medium.

Preparation of ^3H-labelled proteoglycan

Proteoglycan aggregates or proteoglycan monomers from pig laryngeal cartilage or from bovine nasal cartilage were purified as described by Hascall & Sajdera (1969), Sajdera & Hascall (1969) and Hardingham (1976). Their reductive methylation was performed by the method of Means & Feeney (1968), modified as follows. Stock proteoglycan was diluted to 0.55 mg of protein/ml in 0.2 M-sodium borate buffer, pH 9, at 0°C. NaB^3H_4 (2.75 mg, 25 mCi) was added with gentle stirring to 100 ml of that solution, followed by five consecutive additions of 200 μl of aq. 0.5% (w/v) formaldehyde at 5 min intervals. After 30 min the reaction mixture was transferred to dialysis tubing, extensively dialysed at 4°C against water and stored at −20°C until use. A specific radioactivity of about 4×10^6 d.p.m./mg of uronic acids (i.e. about 8×10^6 d.p.m./mg of protein) was achieved.

Preparation of ^{14}C-labelled collagen

^{14}C-labelled salt-soluble collagen (2×10^4–6×10^4 d.p.m./mg) was purified as described previously (Vaes, 1972) from the skin of guinea pigs that had received [^{14}C]glycine intraperitoneally. Acid-soluble collagen, extracted from guinea-pig skin with 0.1 M-acetic acid, was purified in the same way and ^{14}C-labelled (2.5×10^5 d.p.m./mg) by acetylation (Klimman & Karush, 1967). Both collagen preparations were freeze-dried and stored at −20°C until use.

Coating of plates with [^3H]proteoglycan and [^{14}C]-collagen

Salt-soluble collagen (2 mg/ml) was dissolved under gentle stirring in 0.42 M-NaCl/4.2 mM-sodium phosphate buffer/0.2% NaN_3, pH 7.4, at 4°C, and acid-soluble collagen (2 mg/ml, in 0.1 M-acetic acid) was dialysed extensively against 4 × 125 vol. of the same buffer. Stock solutions of proteoglycan monomers or proteoglycan aggregates were prepared in 4.2 mM-sodium phosphate buffer/0.2% NaN_3, pH 7.4, at the concentration (expressed as uronic acid content) of 0.8 mg/ml. Just before the plates were coated, they were mixed at 0°C with collagen and 4.2 mM-phosphate buffer/0.2% NaN_3, pH 7.4; the final concentration of collagen was 666 μg/ml, and that of proteoglycan was 135 μg of uronic acid/ml. Portions of the mixture were pipetted into sterile Multiwell plates (0.2 ml/16 mm-diam. well) and spread over their surface. The plates were incubated at 37°C for at least 96 h to allow the reconstitution of the proteoglycan/collagen gels (Toole & Lowther, 1968; Oegema *et al.*, 1975; Toole, 1976) and their drying. Dried plates could be stored for several weeks at 25°C. Plates used for cell cultures were prepared in the same way, but collagen and proteoglycan were dissolved in sterile salt solutions without NaN_3. Occasionally, plates were coated in the same way with proteoglycan only, by replacing the collagen of the mixture by its solvent buffer.

Assay of proteoglycan- and collagen-degrading enzymes with substrate-coated plates

To remove the unbound proteoglycan and collagen molecules, the plates were preincubated for 24–48 h at 37°C, either (for assays on enzyme solutions) with phosphate-buffered saline containing 0.2% NaN_3, or (for cell cultures) with culture medium containing ten times its usual concentrations of antibiotics. The plates were then washed extensively, respectively with NaN_3 (0.2%)-containing or with sterile phosphate-buffered saline. This procedure usually provided sterile plates, suitable for long-term cultures or incubations. The enzyme solution or the cells to be cultured were then introduced in their appropriate medium (1–2 ml/well) and the plates were incubated at 37°C under the appropriate gas atmosphere. Whenever collagen- or proteoglycan-degrading enzyme activities were assayed in conditioned culture media, their latent forms were first completely activated by trypsin (Vaes, 1972; Huybrechts-Godin & Vaes, 1978) before their introduction into the substrate-coated wells. Collagenase and neutral proteinase (assayed on casein) units are as given by Vaes *et al.* (1978).

The degradation of the proteoglycan and collagen molecules bound to the plates was followed by the release of soluble ^3H- and ^{14}C-labelled material. Double counting of ^3H and ^{14}C radioactivity was performed with a LS-3100 Beckman liquid-scintillation counter, by using 50 μl aqueous samples mixed with 1 ml of Biofluor scintillation liquid. The degradations were expressed as a percentage of the total amounts of ^3H (proteoglycan) or ^{14}C (collagen) present on the plates; 100% values of degradation were determined by incubating the plates with trypsin (25 μg/well) and crude bacterial collagenase (4 mg/well). Blank values, corresponding to the amounts of ^3H or ^{14}C radioactivity spontaneously released from the plates in the absence of enzyme or cells (see below), were subtracted from the percentage release achieved in their presence and the results were normalized by expressing that difference as a percentage of the difference obtained between the 100%-degradation value and the blank.

Characterization of the ^3H-labelled proteoglycan preparations and their degradation products by gel filtration

Samples (1 ml, in eluent) of [^{3}H]proteoglycan, containing 1 mg or 3 mg of uronic acid respectively for proteoglycan aggregates or for proteoglycan monomers, were chromatographed on a Sepharose CL-2B column (1.6 cm × 90 cm; 6–7 ml/h; 3.5–4 ml fractions) equilibrated and eluted at 4°C with sterile 0.5 M-sodium acetate, pH 7. Similar samples of either intact or trypsin-digested proteoglycan were chromatographed on Sepharose CL-6B columns (1.6 cm × 90 cm; 7–8 ml/h; 3.5–4 ml fractions), equilibrated and eluted at 4°C with sterile 0.05 M-sodium acetate, pH 5.8, containing 4 M-guanidinium chloride. Digestions with trypsin (diphenylcarbamoyl chloride-treated) were done at 37°C in 1 ml of 0.1 M-sodium acetate/0.1 M-Tris/HCl, pH 7.3. Samples of proteoglycan aggregates (1 mg of uronic acids) were digested for 0.5 h by 9 μg of trypsin or for 15 h by 45 μg of trypsin, and samples of proteoglycan monomers (3 mg of uronic acids) for 0.5 h by 20 μg of trypsin or for 15 h by 110 μg of trypsin.

Column effluents were analysed for ^{3}H radioactivity, protein (Lowry *et al.*, 1951) and uronic acids (Bitter & Muir, 1962). When 0.05 M-sodium acetate, pH 5.8, containing 4 M-guanidine hydrochloride was used as eluent, protein and uronic acids were determined after dialysis. The void volumes and the total volumes of the columns were measured by chromatography of 1 ml of eluent containing 2 mg of Blue Dextran and 0.05 μCi of $Na_2{}^{35}SO_4$.

Proteoglycan-collagen-coated plates were also digested for various lengths of time with appropriate enzyme solutions as described above. A 0.5 ml sample of the digest, containing the ^{3}H-labelled soluble degradation products of proteoglycan, was chromatographed on a Sepharose 6B column (1.6 cm × 10 cm; 0.32 ml/min; 1 ml fractions) equilibrated and eluted at 4°C with phosphate-buffered saline. The ^{3}H radioactivity was then measured in the various fractions.

Results

Characterization of the ^3H-labelled proteoglycan preparations obtained by reductive methylation

When chromatographed on Sepharose CL-2B, [^{3}H]proteoglycan aggregates were excluded from the gel (Fig. 1*a*), whereas [^{3}H]proteoglycan monomers were eluted as a broad retarded peak (results not shown). Proteoglycan aggregates were thus not irreversibly dissociated by the reductive methylation procedure. After gel filtration of the [^{3}H]proteoglycan aggregates (Fig. 1*b*) or [^{3}H]proteoglycan monomers (results not shown) on Sepharose CL-6B under dissociative conditions, i.e. in the presence of 4 M-guanidinium chloride, the ^{3}H label was mainly recovered together with most of the uronic acids and proteins in the excluded peak, suggesting that it has been largely incorporated into the proteoglycan monomers. The main ^{3}H peak obtained from gel filtration of proteoglycan aggregates amounted to about 78% of the total recovered ^{3}H. It was followed by a small well-included ^{3}H peak (about 13% of the total recovered ^{3}H) that presumably corresponded to the link proteins present within proteoglycan aggregates. Only a minor part (about 4%) of the label, which was eluted at about the total volume of the column, was dialysable (= diffusible); that part increased slowly with increasing storage of the stock [^{3}H]proteoglycan solutions.

After extensive (15 h) trypsin digestion of [^{3}H]-proteoglycan aggregates (Fig. 1*d*) or proteoglycan monomers (results not shown), most of the ^{3}H label (68%) became dialysable. The greater part of the label was eluted slightly ahead of the total volume of the Sepharose CL-6B column with low-molecular-weight fragments that were devoid of uronic acids. Only a minor part was eluted together with most of the uronic acids, i.e. in the fractions containing the large chondroitin sulphate fragments released from proteoglycan by trypsin (Heinegård & Hascall, 1974). The ^{3}H peak corresponding to the link proteins of the proteoglycan aggregates was almost unchanged. A minor ^{3}H peak, corresponding possibly to non-digested proteoglycan aggregates, was excluded.

Fixation of [^3H]proteoglycan and [^{14}C]collagen at the surface of the plates

Plates coated under our working conditions with only [^{3}H]proteoglycan aggregates or [^{3}H]proteoglycan monomers, in the absence of collagen, released respectively 50% or 70% of their radioactivity during the preincubations and the subsequent extensive washings. This contrasted with the more important (85–95%) fixation of the ^{14}C-labelled reconstituted collagen fibres on the plates. The attachment of proteoglycan to the plates was increased in the presence of collagen. As a rule, about 95% of the ^{14}C of the salt-soluble collagen or 85% of the ^{14}C of the acid-soluble collagen, and, respectively, 70–75% of the ^{3}H of the proteoglycan aggregates or 45–50% of the ^{3}H of the proteoglycan monomers, remained fixed at the surface of the plates after the removal of the unbound collagen and proteoglycan by the preincubation and the washings. Within a batch of simultaneously prepared plates, the collagen and proteoglycan contents did not vary by more than 1–2% from one plate to another.

Incubation of the coated plates at 37°C under the conditions of the cultures or of the enzyme assays resulted in the release of some radioactivity into the

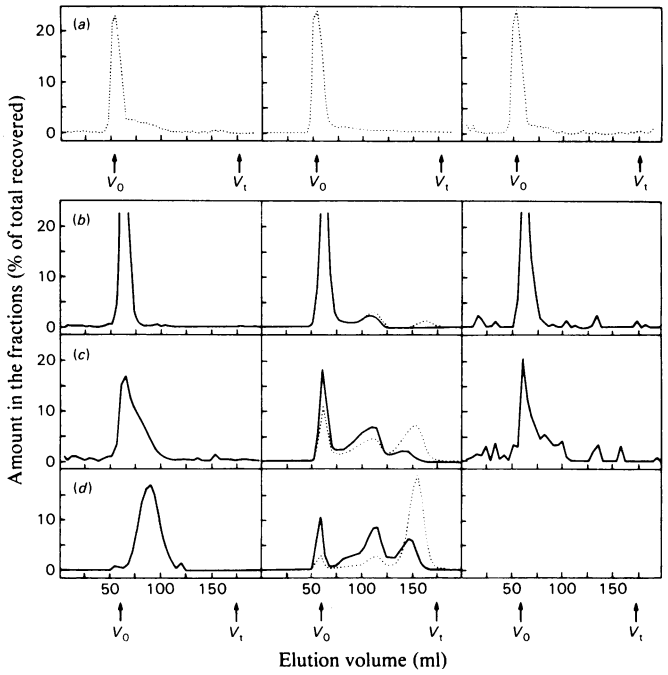

Fig. 1. *Gel chromatography of ^{3}H-labelled proteoglycan aggregates on Sepharose CL-2B or CL-6B*
Samples of [^{3}H]proteoglycan aggregates (pig) were chromatographed as described in the Materials and methods section either on Sepharose CL-2B under associative conditions (no guanidinium chloride) (*a*) or on Sepharose CL-6B, under dissociative conditions, in 4 M-guanidinium chloride (*b*, *c* and *d*). Column effluents were analysed for uronic acids (left), ^{3}H radioactivity (centre) or protein (right), either before dialysis (broken line) or after dialysis (continuous line). V_0, void volume; V_t, total volume. (*a*) and (*b*), [^{3}H]proteoglycan aggregates (almost 100% non-dialysable ^{3}H radioactivity); (*c*) [^{3}H]proteoglycan aggregates digested for 30 min with trypsin (53% non-dialysable ^{3}H radioactivity); (*d*) [^{3}H]proteoglycan aggregates digested for 15 h with trypsin (32% non-dialysable ^{3}H-radioactivity).

surrounding medium or buffer. At a constant ionic strength of about 0.15 mol/l, these 'blank' values were not much affected by the pH (between pH 3.7 and 8) or the buffer composition (Tris/HCl, sodium acetate, formate, cacodylate or phosphate). Moreover, the stability of the proteoglycan/collagen plates prepared with proteoglycan aggregates or proteoglycan monomers was similar. For typical plates, prepared with various batches of [^{14}C]-collagen (either salt-soluble or acid-soluble) and [^{3}H]proteoglycan, the blanks amounted, after 2 and 7–10 days incubation respectively, for ^{14}C, to 2–5% and 5–15%, and for ^{3}H, to about 10% and 20%, of the initial radioactivity fixed on the plates.

Enzymic degradation of the ^{3}H-labelled proteoglycan of the plates

Trypsin was used as a model enzyme to establish the kinetics of the degradation of proteoglycan in the assay. The percentage of ^{3}H-labelled soluble products released by trypsin was not linearly related to the incubation time or enzyme concentration, but related well to the logarithm of either time (Fig. 2) or enzyme concentration (Fig. 3). Almost superposable linear logarithmic plots were obtained with proteoglycan monomers/collagen plates and with proteoglycan aggregates/collagen plates (results not shown). The range of linearity observed in such logarithmic plots extended usually from 15–25 to 75% of degradation, the curves becoming asymptotic as they approach the 0% and 100% values. Complete trypsin digestion of the plates, however, did not solubilize more than 80–85% of the ^{3}H bound to the plates as [^{3}H]proteoglycan aggregates; the remaining 15–20% became soluble only with the concomitant degradation of the [^{14}C]collagen. When chromatographed on a Sepharose 6B column, the soluble ^{3}H-labelled products obtained after a total trypsin digestion of [^{3}H]proteoglycan aggregates/[^{14}C]collagen plates were eluted as a single peak located slightly ahead of the total volume of the column (results not shown). These products appeared thus similar to those obtained by the direct

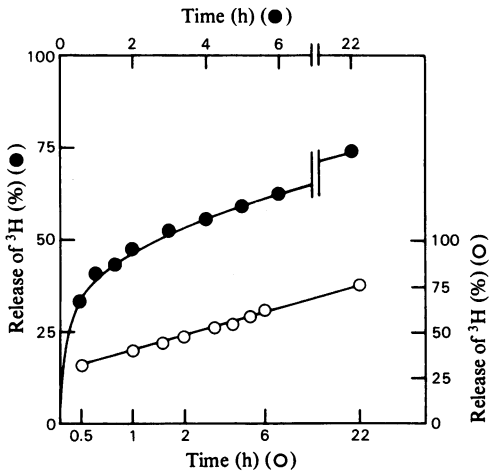

Fig. 2. *Time course of the degradation of proteoglycan monomers by trypsin*

Trypsin (2 μg) was incubated at 37°C for the time indicated in 2 ml of phosphate-buffered saline, pH 7.4, on plates coated with [^{3}H]proteoglycan monomers (bovine) and [^{14}C]collagen (salt-soluble). The values for the percentage release of ^{3}H are plotted against linear (●, upper and left scales) or logarithmic (○, lower and right scales) time scales. Each point is the mean of three determinations.

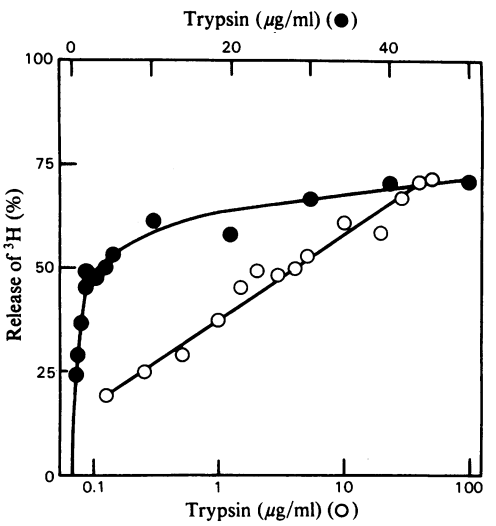

Fig. 3. *Dose–response curve of the degradation of proteoglycan aggregates by trypsin*

The indicated amounts of trypsin were incubated for 2 h under the same conditions as in Fig. 2 on plates coated with [^{3}H]proteoglycan aggregates (pig) and [^{14}C]collagen (acid-soluble). The values for the percentage release of ^{3}H are plotted against linear (●, upper scale) or logarithmic (○, lower scale) scales of trypsin concentrations. Each point is the mean of three determinations.

trypsin digestion of the stock solution of [^{3}H]-proteoglycan aggregates (Fig. 1).

Similar kinetics were obtained with papain (at pH 6.2, in the presence of 10 mM-cysteine) or plasmin (at pH 7.4) as well as with conditioned media from cultures of mouse bone explants or of rabbit fibroblasts (results not shown) known to contain both metal-dependent proteoglycan-degrading neutral proteinase and collagenase activities (Vaes *et al.*, 1978; Huybrechts-Godin & Vaes, 1978). EDTA (5 mM) or heat treatment (15 min at 100°C) completely abolished this action of the fibroblast media.

Chondroitinase ABC [up to 2 units (as defined by the supplier)/well] released only 2–3% of the ^{3}H radioactivity of the proteoglycan aggregates/collagen plates over 54 h of incubation at pH 7.3. Hyaluronidase (50 μg/well) had no significant effect at pH 7.4 on the proteoglycan aggregates. At pH 3.7, it released about 6% of the ^{3}H radioactivity of the proteoglycan aggregates/collagen plates over 48 h: this effect was apparently due to a slight residual proteolytic activity contaminating the hyaluronidase preparation.

Trypsin released also ^{3}H-labelled soluble products from plates coated with proteoglycan aggregates only (results not shown). The concentrations of trypsin needed were then much lower than those required to achieve similar solubilization of ^{3}H-labelled material from proteoglycan/collagen plates. Also, the initial rate of release was linear with time and enzyme concentration. The ^{3}H-labelled products of that trypsin action were, however, not characterized.

Enzymic degradation of the ^{14}C-labelled collagen of the plates

When proteoglycan/collagen-coated plates were incubated at 37°C with high concentrations of trypsin, all their ^{3}H radioactivity was rapidly solubilized, but only a limited fraction of the ^{14}C radioactivity appeared in soluble form in the medium. This fraction varied slightly from one batch of plates to the other. With 50 μg of trypsin/well (2 ml), it amounted typically after 1–6 days of incubation to 15–40% of the [^{14}C]collagen initially present on the plates. This percentage represented the maximum amount of collagen that had been denatured during the preparation of the plates and had become sensitive to the degradative action of trypsin. Therefore trypsin-digestion controls of the plates were included in all series of assays, and degradation of native collagen was considered to occur only when the amount of ^{14}C-labelled material

solubilized from the plates was significantly larger than the maximum amount solubilized by trypsin.

The percentage of ^{14}C-labelled soluble products released from the plates by purified bacterial collagenase was linearly related to the logarithm of either time (Fig. 4*a*) or enzyme concentration (Fig. 4*b*). This collagenase preparation was devoid of significant proteoglycan-degrading activity, as established by the lack of effect of 2 μg of collagenase/ml on the viscosity of solutions of proteoglycan monomers (5.5 mg/ml) over 20 h incubation at pH 7.4 and 37°C, as well as by a minimal (approx. 5%) release of ^{3}H-labelled soluble products achieved from plates coated with proteoglycan aggregates only over 24 h incubation with 5 μg of collagenase/ml. It caused, nevertheless, the release in soluble form of part of the ^{3}H radioactivity bound to the proteoglycan of the plates: the percentage of ^{3}H-labelled soluble products released from [^{3}H]-proteoglycan aggregates amounted approximately to one-fifth of the percentage of ^{14}C-labelled products simultaneously solubilized by the collagenase (Fig. 4), but it amounted to two-thirds of that percentage when [^{3}H]proteoglycan monomers were used to coat the plates (results not shown).

Experiments with conditioned media of rabbit fibroblasts or of mouse bone explants (results not shown), which contain both a proteoglycan-degrading neutral proteinase and collagenase (Vaes *et al.*, 1978; Huybrechts-Godin *et al.*, 1979), allowed us to follow the simultaneous degradation of the two components, proteoglycan and collagen, coating the plates. With both types of media, the percentage of ^{14}C radioactivity solubilized from the collagen was also linearly related to the logarithm of collagenase concentration. Almost all the ^{14}C radioactivity solubilized from the plates was in a dialysable form.

Use of the plates for the direct evaluation of the capacity of synovial cells in culture to degrade proteoglycan and collagen

Cells can be cultured on proteoglycan/collagen-coated plates so as to allow the direct evaluation of their capacity to degrade proteoglycan and collagen. When cultured on the plates, synovial cells obtained from either normal or arthritic rabbits degrade almost simultaneously the two substrates after a lag period of 1–2 days (Fig. 5). They keep these properties after several (at least eight) passages, although their activity may decrease with increasing number of passages. Addition of cycloheximide (2 μg/ml) to the culture medium inhibits these degradations completely (results not shown). Cell-free conditioned media obtained from cultures of synovial cells (but not extracts or homogenates of

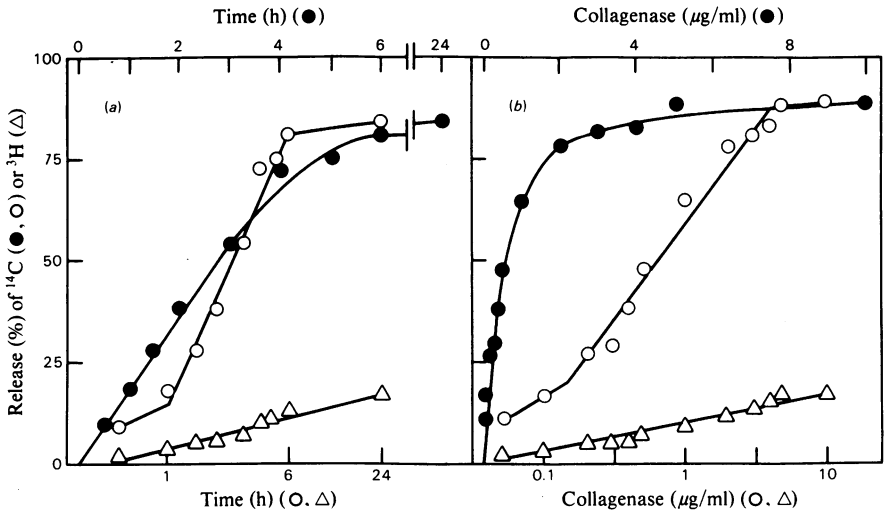

Fig. 4. *Time-course and dose–response curve of the degradation of proteoglycan aggregates and collagen by bacterial collagenase*

In (*a*), purified bacterial collagenase (1 μg) was incubated at 37°C for the time indicated on plates coated with [^{3}H]proteoglycan aggregates (pig) and [^{14}C]collagen (acid-soluble) in 1 ml of 50 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 5 mM-$CaCl_2$ and 0.2 mg of NaN_3/ml. In (*b*), the indicated amounts of purified bacterial collagenase were incubated for 3 h on similar plates under the same conditions. The values for the percentage release of ^{14}C are plotted against linear (●, upper scales) or logarithmic (○, lower scales) time or collagenase-concentration scales, and those for the percentage release of ^{3}H against logarithmic scales only (△, lower scales). Each point is the mean of three determinations.

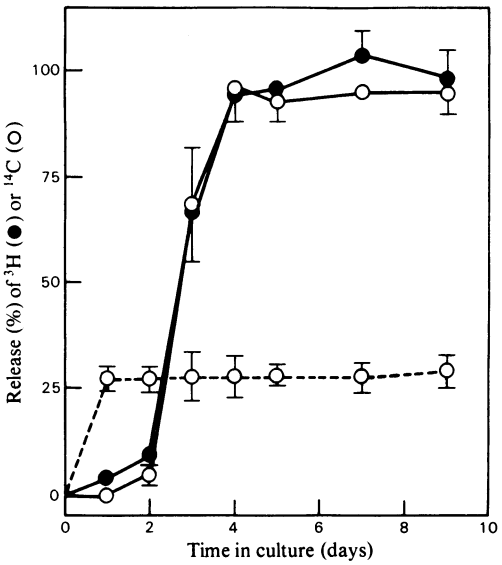

Fig. 5. *Degradation of proteoglycan aggregates and collagen by synovial cells in culture*

Synovial cells (solid lines) from a normal rabbit, obtained after the third passage, were cultivated for the time indicated on Multiwell plates coated with [^{3}H]proteoglycan aggregates (pig) and [^{14}C]collagen (acid-soluble) (10^5 cells in 2ml of medium/well). Control cultures were done without cells, in the presence of 50μg of trypsin (broken line). The releases of soluble ^{3}H-labelled material from proteoglycan (●) and of ^{14}C-labelled material from collagen (○) are expressed as percentages of the total amount of proteoglycan (^{3}H) or collagen (^{14}C) initially present in the wells. Each point is the mean ± S.D. for three cultures.

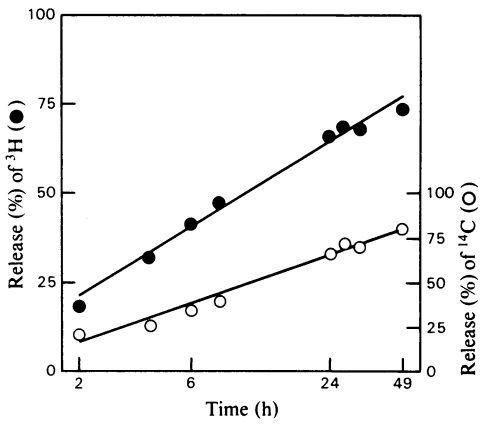

Fig. 6. *Degradation of proteoglycan aggregates and collagen by conditioned medium from synovial-cell cultures*

Conditioned medium (2ml, buffered with 0.1M-sodium cacodylate, pH7) from rabbit synovial-cell cultures, containing 16.4 units of neutral proteinase and 15.2 units of collagenase, were incubated for the time indicated on plates coated with [^{3}H]proteoglycan aggregates (pig) and [^{14}C]collagen (acid-soluble). The values for the percentage release of ^{3}H (●, left scale) or ^{14}C (○, right scale) are plotted against a logarithmic time scale. Each point is the mean of three incubations.

these cells) also degrade the proteoglycan and the collagen of the plates (Fig. 6). Both activities are maximal between pH7 and 8. They are totally inhibited by EDTA (10mM) or *o*-phenanthroline (1mM), but are not significantly affected by Trasylol (1mg/ml), phenylmethanesulphonyl fluoride (1mM), Tos-Lys-CH_2Cl (7-amino-1-chloro-3-L-tosylamido-heptan-2-one, 2.5mM) and 4-hydroxymercuribenzenesulphonate (1mM). Proteoglycan degradation was also almost completely inhibited (approx. 90%) by cysteine (10mM) or foetal calf serum (5%).

Discussion

The new method presented in this paper allows the convenient simultaneous assay of the capacity of cells in culture or of enzyme solutions to degrade the the two major macromolecular constituents of the extracellular matrix of cartilage and other connective tissues, proteoglycan and collagen. It is sensitive, reproducible and suitable for assaying large numbers of samples. Good quantification can be obtained, but it requires careful standardization, particularly in view of the logarithmic dose–response obtained with enzymes acting on either substrate. Similar logarithmic dose–responses have been reported in other assays of proteoglycan-degrading enzymes (Sapolsky *et al.*, 1975; Dingle *et al.*, 1977).

The main advantage of the proposed method over the existing assays is that it allows the direct appraisal of the degradation of both proteoglycan and collagen by living cells in culture. It is thus suitable for an easy evaluation of the production and secretion of the effector proteinases by the cells as well as their regulation by physiological or pharmacological agents. It offers moreover the unique possibility to study proteolytic effects that could be due either to insoluble enzymes associated with the plasma membranes of the cells or to conditions closely dependent on pericellular events. The method is applicable to a large number of experimental situations in the field of connective-tissue degradation. It has already been used extensively in our laboratory under cell-culture conditions with various types of cells: fibroblasts and macrophages (Huybrechts-Godin *et al.*, 1979; Vaes *et al.*, 1980, 1981),

cancer cells (Emonds-Alt *et al.*, 1980) or synovial cells (Fig. 5, and Vaes *et al.*, 1981). It has proved under such conditions to be more convenient, sensitive and reproducible than the method that we used previously (Vaes *et al.*, 1977; Hauser & Vaes, 1978; Huybrechts-Godin & Vaes, 1978) and which involves co-culture of cells with discs of ^{35}S-labelled rabbit ear (or articular) cartilage prepared as described by Ignarro *et al.* (1973). The latter method, however, provides an essential control to ensure that the cells are able to degrade native cartilage proteoglycan and collagen in their real tissue organization.

The method is specific for proteinases degrading either collagen or proteoglycan. The presence of proteoglycan on the plates does not prevent the action of collagenolytic enzymes on collagen, as observed also in another experimental system by Woolley & Evanson (1977). Collagenolysis can be considered as involving native collagen only if it exceeds significantly that achieved by trypsin, which degrades denatured but not native collagen. Therefore plates incubated with high concentrations of trypsin should be included as a control in each series of assay. The method can be used with either salt-soluble or acid-soluble collagen, labelled *in vivo* or *in vitro*, and with either proteoglycan aggregates or proteoglycan monomers. Although proteoglycans can be labelled *in vivo* with ^{35}S before their purification (Dingle *et al.*, 1977), we found it more convenient to label them *in vitro* by reductive methylation (Means & Feeney, 1968), under conditions where the proteoglycan aggregates are stable. This labelling *in vitro* also prevents interference in the assay by sulphatases or glycosidases, as both the glycosaminoglycan chains and the hyaluronate are not appreciably labelled. Indeed, chondroitinase ABC (a glycosidase that degrades the chondroitin sulphate and hyaluronate chains in the proteoglycan; see Muir & Hardingham, 1975) and hyaluronidase had no significant effect on the release of soluble ^{3}H-labelled material from the plates. Also, the results of the gel-filtration experiments on Sepharose CL-6B under dissociative conditions with either intact or trypsin-digested preparations of [^{3}H]proteoglycan aggregates or [^{3}H]proteoglycan monomers are best interpreted as indicating that most of the ^{3}H label is incorporated in the protein core of the proteoglycan monomers. The dissociation observed between the ^{3}H label and the uronic acids on gel filtration of trypsin-digested preparations moreover indicates that, within this protein core, most of the label is associated either with the hyaluronic acid-binding region, a region that contains about one-third of the total proteoglycan protein and has no chondroitin sulphate (Heinegård & Hascall, 1974), or more likely, in view of the known resistance of the hyaluronic acid-binding region to trypsin (Heinegård & Hascall, 1974), either with the peptide regions separating the groups of glycosaminoglycan chains on the chondroitin sulphate attachment region (Roughley & Barrett, 1977) or with the peptide part of the keratan sulphate-rich region (Heinegård & Axelsson, 1977). Whatever the precise localization of the label may be, it is clear from our investigations that a significant solubilization of the label from [^{3}H]-proteoglycan-coated plates is achieved only by their proteolytic treatment. Several proteinases were found to be active on the proteoglycan (monomers or aggregates) of the plates, including the recently discovered proteoglycan-degrading metal-dependent neutral proteinase produced by mouse bone (Vaes *et al.*, 1978), rabbit fibroblasts (Huybrechts-Godin & Vaes, 1978) or rabbit bone-marrow macrophages (Hauser & Vaes, 1978).

The method has, however, an inherent limitation, owing to the fact that the plates are coated with a mixed gel of proteoglycan and collagen (Toole & Lowther, 1968; Oegema *et al.*, 1975; Toole, 1976) that has been slowly dried on its surface. In these gels, proteoglycan is bound to collagen through non-covalent, presumably mainly electrostatic, interactions (Lindahl & Höök, 1978). The relationships existing on the plates between proteoglycan and collagen after the drying of the gels are unknown. Larger amounts of proteoglycan (about 50% more) were fixed on the plates when mixed gels of [^{3}H]-proteoglycan aggregates or [^{3}H]proteoglycan monomers and collagen were dried on their surface than by the drying of solutions containing only proteoglycan, and more proteoglycan aggregates were fixed than proteoglycan monomers. However, the almost complete digestion of the [^{14}C]collagen of the proteoglycan/collagen plates by pure collagenase did not release more than approx. 20% of the [^{3}H]proteoglycan aggregates bound to the plates (Fig. 4), but it released 65–70% of the bound ^{3}H when the plates were coated with [^{3}H]proteoglycan monomers and collagen (results not shown). False positive results for proteoglycan-degrading activity will thus result from collagen degradation by collagenase, presumably owing to the release in soluble form of intact proteoglycan molecules that were adhering to the plates only through interaction with collagen. Plates coated with collagen and proteoglycan monomers should thus be avoided whenever important collagen degradation goes along with proteoglycan digestion. With plates coated with collagen and proteoglycan aggregates, the false positive proteoglycan degradation should be suspected whenever the releases of ^{3}H- and ^{14}C-labelled soluble products occur concomitantly and in a fixed ratio (when expressed as a percentage of the total amount of ^{3}H or ^{14}C initially present) of about 1:5. The artefact could then easily be detected by another assay, e.g. viscometric

(Woessner, 1973), or by using plates coated with proteoglycan only. In our experience, the 'proteoglycan only' plates are suitable for the assay of enzymes in solution, but they provide much more variable and less reliable results than do the proteoglycan/collagen plates when they are used with living cells in culture. Under situations where both collagenase and proteoglycan-degrading proteinase are active, the minimal amount of degraded proteoglycan could also be computed by subtraction of a ^{3}H blank obtained by reference to standards relating the relative amounts of ^{14}C and of ^{3}H released from the plates under the sole action of pure collagenase.

This limitation, however, does not detract from the usefulness of the method in most experimental situations. In our experience, cells (such as macrophages or tumour cells) may produce proteoglycan-degrading proteinase in the absence of collagenase and no false-positive collagenase activity will be detected provided that the required trypsin controls are done. Moreover, several cell systems, such as fibroblasts alone (Fig. 1 in Vaes *et al.*, 1981) or fibroblasts under macrophage stimulation (Huybrechts-Godin *et al.*, 1979; also Fig. 1 in Vaes *et al.*, 1980), degraded proteoglycan before degrading collagen, and no false-positive proteoglycan-degrading activity could occur in this sequence of events.

The use of the assay allowed us to establish, in the present paper, that the production of collagenase by synovial cells (Dayer *et al.*, 1976) goes along with the production of an enzyme that degrades the protein core of cartilage proteoglycan. Both activities are produced by cells obtained either from normal rabbits or from rabbits having an experimental type of immune arthritis (Cooke & Jasin, 1972). Although the enzymes recovered from media conditioned by synovial-cell cultures are mainly latent (Dayer *et al.*, 1976; C. Peeters-Joris & G. Vaes, unpublished work), the direct culture of the cells on the plates allowed us to establish that living cells degrade both substrates under culture conditions, either because the latency of the enzymes was established after their action on the substrates (for instance, by association with an inhibitor) or because part of the latent enzyme precursors was activated *in situ*. Similar observations had previously been made in preliminary experiments (Vaes *et al.*, 1977; also in Fig. 2 in Vaes *et al.*, 1981) when synovial cells were cultured with ^{35}S-labelled cartilage discs. As shown in the present work, these degradations are blocked by the addition of cycloheximide to the cultures, suggesting that the secreted enzymes are products of a new protein synthesis. Indeed, no stored activity could be found in homogenates of the tissue. Medium conditioned by synovial-cell cultures contained both neutral collagenase and a proteoglycan-degrading activity that was inhibited by metal-binding agents, but not by inhibitors of serine proteinases nor by thiol-blocking agents. This activity should be further characterized. It is most likely to be due to the metal-dependent neutral proteinase active on the protein core of proteoglycan that was found in our previous work to be secreted by mouse bone explants (Vaes *et al.*, 1978) and by rabbit macrophages (Hauser & Vaes, 1978) or fibroblasts (Huybrechts-Godin & Vaes, 1978). Presumably that enzyme may play, together with collagenase, a critical role in the degradation of articular cartilage that occurs under the rheumatoid synovial pannus.

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