Degradation of Cartilage Proteoglycans by a Neutral Proteinase Secreted by Rabbit Bone-Marrow Macrophages in Culture

By PIERRE HAUSER and GILBERT VAES

Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, Avenue Hippocrate 75, B-1200 Bruxelles, Belgium

(Received 1 August 1977)

When cultivated together with pieces of cartilage biosynthetically labelled with ^{35}S in their proteoglycans, rabbit macrophages, differentiated *in vitro* from bone-marrow cells, cause the release of soluble ^{35}S -labelled material into the culture medium. This process is inhibited by killing the macrophages or by cycloheximide treatment, and is due to the secretion by the cells of a metal-dependent neutral proteinase capable of degrading cartilage proteoglycan subunits into fragments of high molecular weight. Enzyme activity is optimum at about pH 7, and is inhibited by EDTA, *o*-phenanthroline, cysteine or serum, but not by di-isopropyl phosphorofluoridate nor by 4-hydroxymercuribenzoate. The effect of EDTA is partially reversed by Co^{2+} or Zn^{2+} ions. The enzyme is eluted from Sephadex G-150 columns as a single peak of material (apparent mol.wt. 17000) that contains also most of the proteolytic activity exerted by culture media on Azocoll (denatured collagen) or on casein. The possible role of this metalloproteinase in chronic inflammatory processes is discussed, particularly in connection with joint erosions in rheumatoid arthritis.

Macrophages play an essential role in chronic inflammatory processes, owing to their phagocytic activity, participation in immunological events (Van Furth, 1975; Nelson, 1976) and secretory function (Davies & Allison, 1976; Unanue, 1976). An involvement in the pathophysiology of rheumatoid arthritis is indicated by their abundance in the rheumatoid synovial pannus (Fassbender, 1975). In this disease, articular cartilage is destroyed mainly by contact with inflammatory synovial membrane (Chaplin, 1971; Krane, 1974), apparently owing to lysis of extracellular matrix by infiltrating effector cells, mainly macrophages (Kobayashi & Ziff, 1975). These cells presumably exert their lytic action by secretion of enzymes able to degrade proteoglycans and collagen of the cartilage matrix (Barrett, 1975; Harris, 1976).

To investigate the biochemical mechanisms underlying these phenomena, we developed models *in vitro* (Vaes *et al.*, 1977) in which cells are cultivated in contact with pieces of rabbit cartilage biosynthetically labelled with ^{35}S -labelled proteoglycans. The degradation of the proteoglycans and of collagen of the cartilage matrix is followed by the release of soluble ^{35}S -labelled material and of hydroxyproline. Rabbit macrophages, differentiated *in vitro* from bone-marrow cells, were among the cells studied. In the present paper, we report the occurrence of a rapid degradation of cartilage proteoglycans due to the

synthesis and secretion of a metal-dependent neutral proteinase (EC 3.4.24.–) by the macrophages.

Part of the work described has been presented in preliminary form (Hauser & Vaes, 1977; Vaes *et al.*, 1977).

Experimental

Animals

Male rabbits of the Termonde White strain (De Roskam Breeding, Ghent, Belgium), aged 1–2 months and weighing 1–1.5 kg, were used as a source of bone-marrow cells and of ^{35}S -labelled cartilage.

Isolation and cultivation of rabbit bone-marrow macrophages

Rabbit bone-marrow cells were cultured as described in detail elsewhere (Hauser & Vaes, 1978) in a water-saturated atmosphere of air/ CO_2 (9:1) in Dulbecco's modified Eagle's medium (Smith *et al.*, 1960) containing 1 g of glucose/litre, penicillin (10^5 international units/litre) and streptomycin (100 mg/litre). This medium (referred to as 'Dulbecco's medium' within this paper) was supplemented with L-asparagine (36 mg/litre), folic acid (2 mg/litre) and Trypticase (1.5 g/litre) so as to constitute our 'basal' culture medium. Heat-inactivated (30 min at 56°C) serum, supplemented with 12.5 mM- NaHCO_3 (referred to as 'serum' below), was usually added to this

Abbreviation used: Tos-Lys- CH_2Cl , 7-amino-1-chloro-3-L-tosylamidoheptan-2-one.

basal medium. The cells were first cultured for 6 days in the presence of 15% (v/v) foetal calf serum and of 35% (v/v) horse serum. Non-adherent mononuclear phagocytes were collected and cultured for 24 h in the basal medium supplemented with 15% foetal calf serum. Under these conditions, there were no fibroblasts in the culture system as shown by Hauser & Vaes (1978). The cells were then sedimented and resuspended in the Dulbecco's medium, washed twice by repeated sedimentation (about 1300g-min) and resuspension in that medium and counted in a haemocytometer by using Trypan Blue as diluent.

Preparation of ^{35}S -labelled cartilage substrate

Cartilage discs (about 5 mm diameter, 0.25 mm thickness and 7 mg wet wt.), labelled with ^{35}S in proteoglycans (about 10000 c.p.m./disc) were prepared as described by Ignarro *et al.* (1973) from the ear cartilage of rabbits injected intravenously with 1 mCi of $\text{Na}_2^{35}\text{SO}_4$ 20 h before death. For some experiments, shavings of articular ^{35}S -labelled cartilage were also obtained from either the hip or the knee joints. The cartilage cells were then killed by five successive cycles of freezing and thawing. The cartilage discs were washed extensively in phosphate-buffered saline (8 mM- Na_2HPO_4 /1 mM- KH_2PO_4 /0.14 M- NaCl , pH 7.4) containing penicillin (10^5 international units/litre) and streptomycin (100 mg/litre) and stored in that buffer at -20°C . They were thawed just before use and then, unless otherwise indicated, heated for 30 min at 60°C to lessen autolysis under long-term incubations of ^{35}S -labelled proteoglycans by enzymes present in the tissue.

Degradation of the ^{35}S -labelled cartilage discs by macrophages in culture

Suspensions of non-adherent mononuclear phagocytes (1×10^4 – 5×10^5 cells) in 0.5 ml of Dulbecco's medium were incubated at 37°C with one ^{35}S -labelled cartilage disc in glass conical culture tubes in water-saturated air/ CO_2 (9:1). Blanks containing only the cartilage discs in 0.5 ml of the Dulbecco's medium were run in parallel to allow evaluation of the autolysis of the main components, proteoglycans and collagen, of the cartilage matrix. After 0.5–11 days of culture, 0.25 ml of the culture medium was added to 0.5 ml of 6 M-HCl before the addition of 10 ml of scintillation liquid (Biofluor). The cartilage disc was washed with phosphate-buffered saline and digested for 30 min at 100°C in 1 ml of 6 M-HCl; 0.25 ml of Dulbecco's medium and 10 ml of scintillation liquid were then added to 0.5 ml of the cartilage digest. The radioactivity of both samples was measured in a liquid-scintillation counter and the amount of ^{35}S -labelled fragments released from the cartilage proteoglycans by the action of the macrophages was

expressed as a percentage of the total amount of ^{35}S initially present in the cartilage. In some experiments, the degradation of the proteoglycans was also monitored by the release of uronic acids from the cartilage by assaying them (Bitter & Muir, 1962) in the culture fluid and in the cartilage digest.

Collagen degradation was measured by the loss of hydroxyproline from the cartilage over the culture period. This was done by assaying hydroxyproline (Bergman & Loxley, 1963) in 0.1 ml of either culture medium or cartilage digest after complete hydrolysis in 1 ml of 6 M-HCl at 110°C during 22 h and by expressing the amount of collagen fragments released in the medium as a percentage of the amount of collagen initially present in the cartilage. There was no significant autolysis of the collagen over 5 days' culture of non-heated cartilage discs; this collagen was also resistant to trypsin treatment (25 $\mu\text{g}/\text{ml}$ during 4 days at 37°C). After heating the cartilage for 30 min at 60°C , 20–30% of the total hydroxyproline of the tissue could be released into soluble form by trypsin (see Fig. 1), indicating that part of the collagen had been denatured. Therefore degradation of native collagen was considered to occur only when the amount of hydroxyproline lost from the heat-treated cartilage during the culture or during the assays was significantly larger than the maximum amount solubilized by trypsin.

Assays of a proteoglycan-degrading neutral proteinase in conditioned media from macrophage cultures

Non-adherent mononuclear phagocytes obtained after the first 6 days culture of bone-marrow cells were further cultivated for 14–17 days in basal medium supplemented with 15% foetal calf serum. Under these conditions, the cells adhered rapidly to the wall of the culture vessel (Hauser & Vaes, 1978). Adherent cells (4×10^6 – 5×10^6 cells/ 75 cm^2) were then washed and further cultivated in 10 ml of basal culture medium without serum for successive periods of 3–4 days. The conditioned media collected at the end of each period were centrifuged (about 1300g-min) to discard floating dead cells, buffered by the addition of 0.25 ml of 1 M-sodium cacodylate/HCl buffer, pH 7, and stored at -20°C .

For the assays, one ^{35}S -labelled cartilage disc was incubated for 4–16 h at 37°C together with 0.25 ml of conditioned medium made as above and 0.25 ml of 0.5 M-sodium cacodylate/HCl buffer, pH 7, containing 0.4 mg of NaN_3/ml when the incubation time was 6 h or more. All assays were done in triplicate. For the blanks, the discs were incubated with 0.25 ml of non-cultivated, fresh culture medium in place of the conditioned medium. Neutral proteinase activity was evaluated as described in the preceding section by measuring the amount of ^{35}S -labelled fragments released from the ^{35}S -labelled cartilage proteoglycans

during incubation and expressed as a percentage of the total amount of ^{35}S initially present in the cartilage discs. The results were sometimes expressed in units of activity, one unit referring to the amount of enzyme that releases 1% of the ^{35}S radioactivity initially present in the cartilage/h.

For some experiments, the proteoglycan-degrading proteinase activity was assayed viscometrically by following at 37°C the decrease in specific viscosity ($\eta_{\text{sp.}}$) of a solution of proteoglycan subunits under conditions in which the initial slope of the curve relating $\eta_{\text{sp.}}$ to the time of incubation was proportional to the amount of enzyme preparation (X. Emonds-Alt & G. Vaes, unpublished work). The proteoglycan subunits were purified from bovine nasal cartilage as described by Hascall & Sajdera (1969).

Other enzyme assays

Neutral collagenase (EC 3.4.24.7) assays were done on soluble native ^{14}C -labelled collagen (20000–60000 d.p.m./mg) at 25°C by the method of Vaes (1972). One unit of collagenase activity refers to the degradation of $1\text{ }\mu\text{g}$ of native soluble collagen/min at 25°C . This corresponds approximately to the degradation of $1\text{ }\mu\text{g}$ of gelified collagen/min at 37°C . The neutral proteinase was also assayed on either [^3H]-acetylated casein or Azocoll (an insoluble denatured collagen complexed with a red azo dye) by the methods of Vaes *et al.* (1978). One unit of proteinase activity refers to the amount of enzyme that degrades (per min) either $1\text{ }\mu\text{g}$ of casein into trichloroacetic acid-soluble material or $1\text{ }\mu\text{g}$ of insoluble Azocoll into soluble fragments. β -Glucuronidase (EC 3.2.1.31) was assayed by measuring the hydrolysis of phenolphthalein β -glucuronidate at 37°C (Vaes & Jacques, 1965); one unit of enzyme activity refers to the decomposition of $1\text{ }\mu\text{mol}$ of substrate/min under optimum conditions. Lysozyme (EC 3.2.1.17) was assayed by measuring the initial rate of lysis of *Micrococcus lysodeikticus* by the method of Gordon *et al.* (1974); its activity is expressed in μg by comparison with a standard of pure egg-white lysozyme.

Gel filtration of conditioned culture medium

Gel filtration of macrophage-conditioned culture medium (3 ml samples) was done at $2\text{--}3^\circ\text{C}$ with columns ($2.5\text{ cm} \times 30\text{ cm}$) of Sephadex G-150 equilibrated with 50 mM-sodium cacodylate/HCl buffer, pH 7, containing 0.15 M-NaCl and 5 mM- CaCl_2 (void volume, 51.2 ml; total bed volume, 141.4 ml; flow rate 0.32 ml/min; 3.2 ml fractions). The column was calibrated by the use of markers including horse heart cytochrome *c* (mol.wt. 12400), α -chymotrypsinogen A (mol.wt. 25000) and bovine serum albumin (mol.wt. 67000).

Characterization of the products of degradation of ^{35}S -labelled cartilage proteoglycans

^{35}S -labelled cartilage discs were digested for various lengths of time with conditioned medium as described above. A 0.5 ml sample of the digest, containing the ^{35}S -labelled soluble degradation products released from the cartilage, was chromatographed on a Sepharose 6B column ($1.6\text{ cm} \times 10\text{ cm}$; 0.38 ml/min; 1 ml fractions) equilibrated and eluted at 22°C with 50 mM-sodium cacodylate/HCl buffer, pH 7, containing 0.5 M-NaCl. The ^{35}S radioactivity was then measured as above in the various fractions. The void volume (10 ml) and the total volume (22.8 ml) of the column were measured by chromatography of 0.5 ml of buffer containing 0.5 mg of Blue Dextran and $0.03\text{ }\mu\text{Ci}$ of $\text{Na}_2^{35}\text{SO}_4$.

Samples of cartilage digest were further degraded with papain (EC 3.4.22.2) or chondroitinase ABC (EC 4.2.2.4) before the chromatography on Sepharose 6B. Digestion with papain was by the method of Heinegård & Hascall (1974) by incubating together for 4 h at 37°C , 0.4 ml of cartilage digest in a total volume of 0.5 ml containing 0.2 M-sodium acetate/acetic acid buffer, pH 6.5, 10 mM-EDTA, 10 mM-cysteine and 1 mg of papain. Digestion with chondroitinase ABC (Yamagata *et al.*, 1968) was done by incubating for 4 h at 37°C and at pH 7.3, 0.4 ml of cartilage digest in a total volume of 0.5 ml containing 0.3 M-sodium acetate, 0.5 mg of bovine serum albumin/ml and 1 unit (as defined by the supplier) of chondroitinase ABC.

Materials

Chondroitinase ABC and crystalline egg-white lysozyme were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.), papain (puriss.) was from Fluka A.G. (Buchs, Switzerland), cycloheximide was from Merck A.G. (Darmstadt, W. Germany), Azocoll was from Calbiochem (La Jolla, CA, U.S.A.), Blue Dextran was from Pharmacia A.B. (Uppsala, Sweden), and $\text{Na}_2^{35}\text{SO}_4$ ($>5\text{ mCi}/\mu\text{g}$ of S) from The Radiochemical Centre (Amersham, Bucks., U.K.). Trasylol was a gift of Bayer A.G. (Wuppertal, W. Germany). Dulbecco's modified Eagle's medium, foetal calf serum and horse serum were from Gibco-Biocult (Glasgow, Scotland, U.K.) and Trypticase (Bacto-tryptic soya broth, dehydrated) was from Difco (Detroit, MI, U.S.A.). Penicillin (crystalline penicillin G) was from Continental Pharma (Brussels, Belgium), streptomycin (streptomycin sulphate), from Laboratoria Wolfs (Antwerp, Belgium) and Biofluor, from New England Nuclear (Boston, MA, U.S.A.).

All the other enzymes and special chemicals used were from the same suppliers as in our previous work (Vaes, 1972; Eeckhout & Vaes, 1977).

Results

Degradation of cartilage by macrophages in culture

Non-adherent mononuclear phagocytes were first cultivated during several days in a medium devoid of serum in the presence of heat-treated ^{35}S -labelled cartilage of either ear or articular origin (Fig. 1). In the presence of living phagocytes, an almost complete degradation of the ^{35}S -labelled proteoglycans of both cartilages occurred over 2–3 days culture (Fig. 1a). There was, however, no degradation of native cartilage collagen, as the cells did not cause the release of more soluble hydroxyproline from the cartilage over 7 days culture than did trypsin (Fig. 1b). The release of soluble ^{35}S -labelled fragments from the cartilage proteoglycans was dependent on the number of cells put into the culture vessels together with the ^{35}S -labelled cartilage discs (Fig. 2a); it occurred after an initial lag phase that was indirectly proportional to the number of cells present. Non-heated ^{35}S -labelled cartilage discs were similarly degraded by living phagocytes, but the extent of autolysis of

Fig. 1. *Degradation of cartilage by macrophages in culture*

Heated ^{35}S -labelled cartilage pieces, either ear (closed symbols) or articular (open symbols), were cultivated for the time indicated in 0.5 ml of medium under three conditions: with 1.3×10^5 non-adherent mononuclear phagocytes ($\circ$, $\bullet$), without cells ($\triangle$, $\blacktriangle$) or, as a further control, in the presence of $5 \mu\text{g}$ of trypsin ($\square$, $\blacksquare$). The release of soluble ^{35}S -labelled material (a) or of hydroxyproline (b) from the cartilage is expressed as a percentage of the total ^{35}S or hydroxyproline present in the cartilage at the beginning of the cultures. Each point is the mean of three cultures.

the cartilage ^{35}S -labelled proteoglycans became considerably more significant in comparison, so as to make demonstration of an action by the cells less convincing (Fig. 2b). The killing of the cells by repeated freezing and thawing as well as the addition of $2 \mu\text{g}$ of cycloheximide/ml to the cultures abolished the capacity of the cells to release soluble ^{35}S -labelled fragments from the cartilage (Fig. 2b).

Fig. 2. *Degradation of the proteoglycans of either heated or non-heated cartilage by live or dead macrophages in culture and effect of cell number and of cycloheximide*

In (a), heated ear ^{35}S -labelled cartilage discs were cultivated for the time indicated in 0.5 ml of medium containing no cells ($\triangle$), 0.4×10^5 ($\blacktriangle$), 0.8×10^5 ($\circ$) or 4×10^5 ($\bullet$) non-adherent phagocytes. The release of soluble ^{35}S -labelled material is expressed as a percentage of the total ^{35}S present in the cultures. Each point is the mean of three cultures. In (b), the results of two experiments are presented. In the first experiment (continuous lines) native (non-heated) ^{35}S -labelled cartilage discs were cultivated for the indicated time in 0.5 ml of medium containing no cells ($\circ$) or 5×10^5 non-adherent phagocytes that were either alive ($\bullet$) or killed by repeated freezing and thawing ($\blacktriangle$). In the second experiment (broken lines), heated (30 min at 60°C) ^{35}S -labelled cartilage was cultivated without cells ($\circ$) or in the presence of 10^5 phagocytes without additive ($\bullet$) or with cycloheximide ($2 \mu\text{g}/\text{ml}$) added to the medium ($\blacksquare$). In both experiments, the degradation of the cartilage proteoglycans was assayed by the release of soluble ^{35}S -labelled material in the culture fluid. Each point is the mean of three cultures.

Secretion of a proteoglycan-degrading enzyme by macrophages in culture

A search was then made for a proteoglycan-degrading enzyme in conditioned media surrounding adherent macrophages in culture. Cell-free media released soluble ^{35}S -labelled material from the cartilage discs indicating that such an enzyme was indeed released by the macrophages. The release was almost linear with time during the first 6 days of culture but levelled off thereafter. The rate of secretion of this enzyme was similar to that of collagenase, whereas that of lysozyme went on linearly with time for 10 days and that of β -glucuronidase for at least 14 days (see Fig. 2 in Hauser & Vaes, 1977). After 3 days of culture (Table 1), insignificant amounts of proteoglycan-degrading enzyme and of collagenase were found within the macrophages; the whole activity was in the culture fluid. This contrasted with the distribution of β -glucuronidase between cells (69%) and medium (31%). Lysozyme was 92% in the medium. The absence of the proteoglycan-degrading enzyme in the cell lysate was apparently not due to the presence of inhibitors of the enzyme within the lysate; indeed, the addition of 1 vol. of cell lysate to a sample of conditioned medium did not modify the enzyme activity present within the medium (results not shown).

Characterization of the proteoglycan-degrading enzyme

The proteoglycan-degrading enzyme present in the conditioned media exerts its optimum activity on ^{35}S -labelled cartilage at about pH 7 (Fig. 3). The quantity of soluble ^{35}S -labelled material released by the enzyme, expressed as percentage of the ^{35}S initially present in the cartilage discs, is directly proportional to the time of incubation or to the amount of enzyme (conditioned medium) added for digestions extending from 5 to 65% of the cartilage ^{35}S (Fig. 4). EDTA inhibits completely the activity of the enzyme at a concentration (0.1 mM) distinctly lower than that of the Ca^{2+} (0.72 mM) or Mg^{2+}

(0.32 mM) present simultaneously in the assays (Table 2). The activity is also inhibited by *o*-phenanthroline, cysteine and foetal calf serum as well as by casein or Azocoll, but not by di-isopropyl phosphorofluoridate, soya-bean trypsin inhibitor, Trasylol, Tos-Lys- CH_2Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 'TLCK') and 4-hydroxymercuribenzoate (Table 2; see also Fig. 7). The enzyme activity lost in the presence of 1 mM-EDTA is partially recovered by the addition of bivalent Co^{2+} or Zn^{2+} ions, possibly also by Mn^{2+} ions, although an excess of these ions is inhibitory; other bivalent ions (Cu^{2+} ,

Fig. 3. Influence of pH on the activity of the proteoglycan-degrading enzyme of macrophage-conditioned culture medium

Incubation of either macrophage-conditioned medium (solid line and solid symbols) or of fresh non-cultivated medium (broken line and open symbols) was done in the presence of heated ^{35}S -labelled cartilage discs in the following 0.25 M buffers: sodium acetate/acetic acid ($\bullet$, $\circ$), sodium cacodylate/HCl ($\blacktriangle$, $\triangle$) and Tris/HCl ($\blacksquare$, $\square$).

Table 1. Distribution of macrophage enzymes between cells and conditioned medium

Adherent rabbit bone-marrow macrophages, obtained after 5 days' culture in basal medium supplemented with 15% foetal calf serum, were further cultivated for 3 days in basal medium without serum. The conditioned medium was then collected and the cell lysate was prepared by scraping the macrophages with a 'rubber policeman' in phosphate-buffered saline containing 0.1% (w/v) Triton X-100; it was verified that complete lysis of the cells was obtained by this treatment. Triton X-100 was added at the same concentration to the conditioned medium before the assays.

Source of enzyme	Enzyme activity (units/ 4×10^6 cells)			
	Proteoglycan-degrading enzyme	Collagenase	Lysozyme	β -Glucuronidase
Cell lysate	0.1	0	6	2.84
Conditioned medium (3 days)	341	7.8	69	1.28

Fig. 4. Linearity of the proteoglycan-degrading enzyme assay with regard to time and enzyme concentration

In (a), heated ^{35}S -labelled cartilage discs were incubated for the times indicated with 0.25 ml of conditioned medium (●) or, for the blanks, of fresh non-cultivated culture medium (▲). In (b), the discs were incubated for 15 h with the indicated volumes of another conditioned medium, the total volume of culture medium being adjusted to 0.25 ml by the addition of fresh non-cultivated medium.

Table 2. Effect of various agents on the proteoglycan-degrading-enzyme activity of macrophage-conditioned media

Macrophage-conditioned medium (0.2 ml) was incubated for 4 h in a total volume of 0.5 ml containing the agent, 0.2 M-sodium cacodylate/HCl buffer, pH 7, and one ^{35}S -labelled cartilage disc. Di-isopropyl phosphorofluoridate was dissolved in propan-2-ol which was present at 250 mM in the assays. The controls (0% inhibition) released 38% of the ^{35}S present in the discs over the incubation period.

Compound	Concn.	Inhibition (%)
EDTA	0.1 mM	96
<i>o</i> -Phenanthroline	1 mM	99
Cysteine	2 mM	98
Serum (foetal calf)	5% (v/v)	100
Casein	10 mg/ml	76
Azocoll	40 mg/ml	75
Propan-2-ol	250 mM	3
Di-isopropyl phosphorofluoridate	2 mM	0
Soya-bean trypsin inhibitor	5 mg/ml	0
Trasylol	1 mg/ml	0
Tos-Lys-CH ₂ Cl	0.5 mM	8
4-Hydroxymercuribenzoate	1 mM	3

Fe^{2+} , Mg^{2+}) have no effect (Fig. 5). Ca^{2+} restores some activity, but only at high concentrations (20–100 mM).

Uronic acids are released together with ^{35}S during the degradation of the cartilage discs. When both are expressed as a percentage of the total amount initially present in the discs, these releases were similar up to about 50% degradation of the cartilage tissue. The soluble ^{35}S -labelled cartilage degradation

products chromatograph on a Sepharose 6B column at a position intermediate between the void volume and the ^{35}S -labelled material obtained by a subsequent papain digestion of these products, well ahead of the ^{35}S -labelled material released from the cartilage digest by chondroitinase ABC (Fig. 6). Moreover, macrophage-conditioned media decrease the specific viscosity of solutions of purified proteoglycan subunits obtained from bovine nasal cartilage, and this effect is also inhibited by EDTA, cysteine or serum (Fig. 7).

A single peak of proteoglycan-degrading enzyme activity was recovered after gel filtration of conditioned culture medium on Sephadex G-150 (Fig. 8, solid curve). By comparison with the results obtained by calibration of the column, the apparent molecular weight of the enzyme was about 17000.

Macrophage-conditioned culture media exert also a proteolytic activity on other protein substrates. They release soluble dyed products from insoluble Azocoll as well as trichloroacetic acid-soluble fragments from casein. These activities, optimum about pH 7, are also inhibited by EDTA, cysteine or serum (Table 3). After gel filtration of the culture medium on Sephadex G-150, the activity exerted on Azocoll was recovered in two peaks; about 65% of this activity was eluted in the peak active on cartilage proteoglycans (Fig. 8, dashed curve). This material contained also about 90% of the proteolytic activity exerted on casein. These data, together with those showing inhibition of the proteoglycan-degrading enzyme by casein or Azocoll (Table 2), suggest that casein and Azocoll (i.e. denatured collagen) may constitute other substrates for the proteoglycan-degrading enzymes.

Fig. 5. Attempts to restore by bivalent cations the proteoglycan-degrading enzyme activity of macrophage-conditioned medium after its inhibition by EDTA

Conditioned medium (0.25 ml) was preincubated for 15 min at 37°C with 1 mM-EDTA and 0.15 ml of 0.5 M-Tris/HCl buffer, pH 7.5 (at 25°C), to inhibit completely the proteoglycan-degrading enzyme activity; controls were preincubated without EDTA. The indicated bivalent cations (0.1 ml) were then added (as their sulphate salts, except for CaCl₂) to the EDTA-treated media to give the final concentration indicated on the abscissa, and 0.1 ml of water was added to the controls. The mixtures were further incubated during 4 h at 37°C with heated ³⁵S-labelled cartilage discs. The recovered activities are expressed as a percentage of the controls (100% recovered activity corresponds to the release of 35% of the ³⁵S present in the cartilage discs).

Discussion

The proteoglycan-degrading enzyme that is secreted by rabbit bone-marrow macrophages under our culture conditions can be identified as a neutral proteinase that degrades the protein core of proteoglycan subunits present in cartilage. It causes the release of soluble ³⁵S-labelled material as well as of soluble uronic acids from discs of cartilage biosynthetically labelled with ³⁵S in their proteoglycans. Although this material could possibly be intact proteoglycan subunits released from proteoglycan aggregates (for review, see Rosenberg, 1975) by the degradation of the hyaluronate filamentous backbone or of link glycoproteins, the fact that the enzyme considerably decreases the specific viscosity of solutions of proteoglycan subunits purified from bovine nasal cartilage indicates that the enzyme degrades these sub-

Fig. 6. Gel filtration of the ³⁵S-labelled degradation products of cartilage on Sepharose 6B

The soluble ³⁵S-labelled degradation products (●) were chromatographed, as described in the Experimental section, after digestion of ³⁵S-labelled cartilage by 0.25 ml of macrophage-conditioned medium for 4 (a), 24 (b) or 48 (c and d) h. These digestions corresponded respectively to the release of 47, 71 and 90% of the total ³⁵S label initially present in the cartilage. In experiment (c), chromatographs were done either on the 48 h-cartilage digest (●) or on that digest further degraded by papain (○) or by chondroitinase ABC (▲). In experiment (d), fresh enzyme preparation (0.1 ml of macrophage-conditioned medium) was added to the cartilage digest at 23 and 30 h to ensure that it had been completely digested. V₀, void volume; V_t, total volume of the column.

units into fragments. By chromatography on Sepharose 6B, it appears that the soluble ³⁵S-labelled products of the enzyme action are, however, of high molecular weight. Moreover, they are further degraded into smaller fragments by papain, a proteinase that is known to release from cartilage proteoglycan peptide fragments containing mainly a single polysaccharide chain (Luscombe & Phelps, 1967; Mathews, 1971; Heinegård & Hascall, 1974). They thus cannot be identified with fragments of the polysaccharide chains that could possibly have been produced by the action of endo- or exo-glycosidases, as these fragments would necessarily be smaller than those produced by papain. Low-molecular-weight ³⁵S-labelled fragments, such as the sulphated sugars

Fig. 7. Effect of macrophage-conditioned medium on the viscosity of a solution of proteoglycan subunits

Macrophage-conditioned medium (0.2 ml) was incubated at 37°C within Ostwald viscometers in a total volume of 0.75 ml containing 5 mg of purified proteoglycan subunits (from bovine nasal mm-cartilage) in 77 Tris/HCl buffer, pH 7.4, and 50 mM-NaCl. The specific viscosities (η_{sp}) were calculated from the flow times measured at the times indicated. The conditioned medium was incubated in the absence of any additive (○) or in the presence, in the incubation mixture, of 2 mg of soya-bean trypsin inhibitor/ml (◇), of 0.66 mM- (△) or 2 mM- (▲) cysteine, of 0.66 mM- or 2 mM- (□) EDTA or of 0.67% (v/v) of foetal calf serum (●).

released by chondroitinase ABC or the inorganic sulphate released by a sulphatase, were not found in any significant amount after an exhaustive digestion of the ^{35}S -labelled cartilage by macrophage-conditioned media. Thus only a limited proteolytic degradation of the protein core of the proteoglycan subunits adequately explains our observations. This proteolysis was displayed, throughout the present study, on discs of rabbit ear cartilage that had been heated for 30 min at 60°C to decrease the autolysis of the cartilage proteoglycans under the action of enzymes present in the tissue. This was needed to allow more precise analytical assays of the macrophage enzyme. The heating is, however, not necessary for the action of the macrophage proteinase on the cartilage proteoglycans, as the proteoglycans of native non-heated ^{35}S -labelled cartilage discs were also degraded by the enzyme. Moreover, heated or non-heated ^{35}S -labelled rabbit articular cartilage was equally good as a substrate for the enzyme as rabbit ear ^{35}S -labelled cartilage.

The macrophage proteinase has optimum activity at about pH 7. It is completely inhibited by EDTA (even in the presence of an excess of Ca^{2+} and Mg^{2+} ions), *o*-phenanthroline, cysteine or serum, but not by inhibitors of serine proteinases or by thiol-blocking agents. It can thus be classed among the metal-dependent neutral proteinases. Our attempts to restore the activity of EDTA-inhibited enzyme by the

Fig. 8. Distribution of the proteolytic activities exerted on proteoglycans and on Azocoll after gel filtration of macrophage-conditioned medium on Sephadex G-150

Macrophage-conditioned medium (3 ml) was chromatographed on a Sephadex G-150 column as described in the Experimental section. The proteoglycan-degrading enzyme activity (● and continuous line) was measured on a sample (0.2 ml) of each fraction by incubating it for 25 h with ^{35}S -labelled cartilage discs; its recovery after filtration through the column was 89%. The proteolytic activity on Azocoll (▲ and dashed line) was measured on a 0.5 ml sample of each fraction by incubating it for 3 h at 37°C with 20 mg of Azocoll in a total volume of 1 ml containing 0.1 M-cacodylate buffer, pH 7.0; its recovery was 65%. The enzyme activities are expressed as a percentage of the total amount recovered in the fractions. The arrows indicate the elution peaks of (a) Blue Dextran, (b) serum albumin, (c) α -chymotrypsinogen A, (d) cytochrome *c*.

addition of bivalent cations indicate that either Co^{2+} or Zn^{2+} could be the required metal. The recovery of some activity by the addition of large concentrations of Ca^{2+} ions may possibly be due to a displacement by Ca^{2+} of the Co^{2+} or Zn^{2+} complexed with EDTA. Moreover, other proteins (casein and Azocoll) inhibited the activity of the proteinase on proteoglycans, and evidence was obtained that they may represent other substrates for the enzyme.

The rabbit macrophage metal-dependent proteinase appears similar in several respects to the neutral metalloproteinase, active on proteoglycans, Azocoll or casein, found in the fluid surrounding mouse bone explants in culture (Vaes *et al.*, 1978) as well as to the proteoglycan-degrading proteinase secreted by rabbit fibroblasts in culture (G. Huybrechts-Godin & G. Vaes, unpublished work; Vaes *et al.*, 1977). However, the mouse bone enzyme is present in a latent trypsin-activatable form, but so far we have found no latent activity for the enzyme present in macrophage-conditioned media. The mol.wt. (40000) of the

Table 3. *Proteolytic degradation of Azocoll and casein by macrophage-conditioned medium*

In Expts. A1 and A2, Azocoll (20mg) was incubated for 16h at 37°C in a total volume of 1ml with 20µl of macrophage-conditioned culture medium together with, in Expt. A1, 0.1M-sodium cacodylate/HCl buffer of the indicated pH or, in Expt. A2, 0.1M-cacodylate buffer, pH7.0, either alone or containing EDTA, cysteine or calf serum. In Expt. A2, the Azocoll was, however, added to the incubated mixtures after 15min of preincubation done in its absence. In Expts. B1 and B2, [³H]acetylated casein (0.25mg; about 300000d.p.m./mg) was incubated for 15min at 37°C in a total volume of 100µl together with, in Expt. B1, 0.125M concentrations of sodium acetate/HCl, cacodylate or Tris/HCl buffer of the indicated pH (at 25°C) or, in Expt. B2, 0.075M-cacodylate buffer, pH7.5, either alone or containing EDTA, cysteine or calf serum at the concentrations indicated in the Table. For Expt. B2, the enzyme preparation was first preincubated for 15min at 37°C in the absence of substrate either as such or with 1.3mM-EDTA, 6.6mM-cysteine or 6.6% (v/v) serum. The proteinase activities are reported as percentages of the maximum activities observed within each experiment. The absolute values corresponding to these maximum (100%) activities were 47 units/ml of culture medium for Expts. A1 and A2, 31 units/ml for Expt. B1 and 22 units/ml for Expt. B2.

Expt.	Substrate	Conditions	Proteinase activity (% of maximum)
A1	Azocoll	Cacodylate pH5.8	46
		pH6.2	71
		pH6.7	100
		pH7.1	83
A2	Azocoll	Control (no additive)	100
		+EDTA (1 mM)	3
		+Cysteine (5 mM)	62
		+Serum (5%, v/v)	0
B1	Casein	Acetate, pH5	9
		pH5.5	22
		pH6.1	32
		Cacodylate, pH6.0	39
		pH6.6	48
		pH7.1	69
		pH7.4	97
		Tris, pH7.0 (at 25°C)	73
		pH7.8 (at 25°C)	100
B2	Casein	pH8.2 (at 25°C)	87
		Control (no additive)	100
		+EDTA (1 mM)	2
		+Cysteine (1 mM)	11
		+Serum (5%, v/v)	8

glycans present in human cartilage (Sapolsky *et al.*, 1976), or to a neutral proteinase active on globin which is secreted by rabbit alveolar macrophages in culture and which is inhibited by EDTA (Horwitz *et al.*, 1976). It is, however, distinct from other neutral proteinases secreted by mouse or guinea-pig macrophages: indeed, plasminogen activator (Unkeless *et al.*, 1974) and elastase (Werb & Gordon, 1975*b*) are both serine proteinases, and the secretion of the metal-dependent neutral collagenase (Wahl *et al.*, 1974*a,b*; Werb & Gordon, 1975*a*) does not necessarily accompany that of the proteoglycan-degrading neutral proteinase in our experience. Species differences within the macrophage proteinases may, however, possibly exist.

A property common to both the mouse bone enzyme and the rabbit macrophage proteinase is that they are both apparently not stored within the cells or tissues, but are continuously secreted under culture conditions. The macrophage neutral proteinase shares this property with collagenase, which has a rate of secretion similar to that of the proteinase, and to some extent also with lysozyme, whose rate of secretion is, however, different and which is found in small amounts in cells. Thus the discharge of lysozyme may depend on a distinct secretory mechanism, as observed by Gordon & Werb (1976) for mouse peritoneal macrophages. β-Glucuronidase, a lysosomal enzyme, has also a different rate of secretion and is stored in large amounts inside cells. Thus it appears unlikely that the neutral proteinase and collagenase could be part of the lysosomal content. Moreover, the fact that the release of neutral proteinase is inhibited by killing the cells or by cycloheximide suggests that this enzyme is continuously synthesized by the macrophages just before being secreted.

Macrophages have for a long time been known mainly for their property of phagocytosis, but appear more and more clearly to possess an important secretory function (reviewed by Unanue, 1976). They are able to secrete numerous hydrolytic enzymes, not only lysosomal acid hydrolases (Davies & Allison, 1976) but also lysozyme (Gordon *et al.*, 1974), plasminogen activator (Unkeless *et al.*, 1974), collagenase (Wahl *et al.*, 1974*a,b*; Werb & Gordon, 1975*a*), elastase (Werb & Gordon, 1975*b*) and other neutral proteinases (Gordon & Werb, 1976; Horwitz *et al.*, 1976). The secretion of a neutral metal-dependent proteinase capable of degrading cartilage proteoglycans may now be added to this list. It seems evident that these enzymes must play an important role in the chronic inflammatory processes where macrophages are abundant. This view is supported by the fact that the secretion of several hydrolytic enzymes has been found to be stimulated by agents known to participate in the initiation or the regulation of chronic inflammation, such as lymphokines (Wahl *et al.*, 1974*b*; Pantalone & Page, 1975;

active enzyme from bone is also much larger than that of the rabbit macrophage enzyme. The rabbit macrophage metalloproteinase may be related to the metal-dependent neutral proteinase active on proteo-

Klimetzek & Sorg, 1977), complement factors (Schorlemmer & Allison, 1976; Schorlemmer *et al.*, 1977), endotoxin (Wahl *et al.*, 1974a), immune complexes (Cardella *et al.*, 1974), group A streptococcal cell-wall substance (Davies *et al.*, 1974a), dental plaque (Page *et al.*, 1973), asbestos (Davies *et al.*, 1974b), etc. In this respect, the capacity of the macrophages to secrete a proteinase capable of degrading cartilage proteoglycans at neutral pH may be particularly significant, as is that of a neutral collagenase demonstrated by others, in the erosion of articular cartilage that characterizes rheumatoid and other inflammatory arthritis. The mechanisms controlling this secretion remain, however, to be elucidated, as well as possible pharmacological ways to inhibit either the secretion or the activity of the enzyme.

This work was supported by grants from the Belgian Fonds de la Recherche Scientifique Médicale and from Société Rhône-Poulenc, Paris. We thank Miss M. Loiseau and Mrs. F. Fiévet-Groyne for their excellent technical assistance.

References

- Barrett, A. J. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 189–226, North-Holland Publishing Co., Amsterdam
- Bergman, D. & Loxley, R. (1963) *Anal. Chem.* **35**, 1961–1965
- Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
- Cardella, C. J., Davies, P. & Allison, A. C. (1974) *Nature (London)* **247**, 46–48
- Chaplin, D. M. (1971) *J. Bone Jt. Surg.* **53B**, 711–717
- Davies, P. & Allison, A. C. (1976) in *Immunobiology of the Macrophage* (Nelson, D. S., ed.), pp. 427–461, Academic Press, New York
- Davies, P., Page, R. C. & Allison, A. C. (1974a) *J. Exp. Med.* **139**, 1262–1282
- Davies, P., Allison, A. C., Ackerman, J., Butterfield, A. & Williams, S. (1974b) *Nature (London)* **251**, 423–425
- Eeckhout, Y. & Vaes, G. (1977) *Biochem. J.* **166**, 21–31
- Fassbender, H. G. (1975) *Pathology of Rheumatoid Diseases*, Springer Verlag, Berlin
- Gordon, S. & Werb, Z. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 872–876
- Gordon, S., Todd, J. & Cohn, Z. A. (1974) *J. Exp. Med.* **139**, 1228–1248
- Harris, E. D., Jr. (1976) *Arthritis Rheum.* **19**, 68–72
- Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384–2396
- Hauser, P. & Vaes, G. (1977) *Biochem. Soc. Trans.* **5**, 1091–1093
- Hauser, P. & Vaes, G. (1978) *Exp. Cell Res.* **111**, 353–361
- Heinegård, D. & Hascall, V. C. (1974) *Arch. Biochem. Biophys.* **165**, 427–441
- Horwitz, A. L., Kelman, J. A. & Crystal, R. G. (1976) *Nature (London)* **264**, 772–774
- Ignarro, L. J., Oronsky, A. L. & Perper, R. J. (1973) *Clin. Immunol. Immunopathol.* **2**, 36–51
- Klimetzek, V. & Sorg, C. (1977) *Eur. J. Immunol.* **7**, 185–187
- Kobayashi, I. & Ziff, M. (1975) *Arthritis Rheum.* **18**, 475–484
- Krane, S. M. (1974) *Arthritis Rheum.* **17**, 306–313
- Luscombe, M. & Phelps, C. F. (1967) *Biochem. J.* **103**, 103–109
- Mathews, M. B. (1971) *Biochem. J.* **125**, 37–46
- Nelson, D. S. (1976) *Immunobiology of the Macrophage*, Academic Press, New York
- Page, R. C., Davies, P. & Allison, A. C. (1973) *Arch. Oral Biol.* **18**, 1481–1495
- Pantalone, R. M. & Page, R. C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2091–2094
- Rosenberg, L. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 105–128, North-Holland Publishing Co., Amsterdam
- Sapolsky, A. I., Keiser, H., Howell, D. S. & Woessner, J. F., Jr. (1976) *J. Clin. Invest.* **58**, 1030–1041
- Schorlemmer, H. U. & Allison, A. C. (1976) *Immunology* **31**, 781–788
- Schorlemmer, H. U., Edwards, J. H., Davies, P. & Allison, A. C. (1977) *Clin. Exp. Immunol.* **27**, 198–207
- Smith, J. D., Freeman, G., Vogt, M. & Dulbecco, R. (1960) *Virology* **12**, 185–196
- Unanue, E. R. (1976) *Am. J. Pathol.* **83**, 396–417
- Unkeless, J. C., Gordon, S. & Reich, E. (1974) *J. Exp. Med.* **139**, 834–850
- Vaes, G. (1972) *Biochem. J.* **126**, 275–289
- Vaes, G. & Jacques, P. (1965) *Biochem. J.* **97**, 380–388
- Vaes, G., Hauser, P., Huybrechts-Godin, G. & Peeters-Joris, Ch. (1977) in *Perspectives in Inflammation* (Willoughby, D. A., ed.), pp. 115–126, M.T.P. Press, Lancaster
- Vaes, G., Eeckhout, Y., Lenaers-Claeys, G., François-Gillet, Ch. & Druetz, J. E. (1978) *Biochem. J.* **172**, 261–274
- Van Furth, R. (1975) *Mononuclear Phagocytes in Immunity Infection and Pathology*, Blackwell, Oxford
- Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. & Martin, G. R. (1974a) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3598–3601
- Wahl, L. M., Wahl, S. M., Mergenhagen, S. E. & Martin, G. R. (1974b) *Science* **187**, 261–263
- Werb, Z. & Gordon, S. (1975a) *J. Exp. Med.* **142**, 346–360
- Werb, Z. & Gordon, S. (1975b) *J. Exp. Med.* **142**, 361–377
- Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535