The Simultaneous Release by Bone Explants in Culture and the Parallel Activation of Procollagenase and of a Latent Neutral Proteinase that Degrades Cartilage Proteoglycans and Denatured Collagen

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1. A latent neutral proteinase was found in culture media of mouse bone explants. Its accumulation during the cultures is closely parallel to that of procollagenase; both require the presence of heparin in the media. 2. Latent neutral proteinase was activated by several treatments of the media known to activate procollagenase, such as limited proteolysis by trypsin, chymotrypsin, plasmin or kallikrein, dialysis against 3 M-NaSCN at 4°C and prolonged preincubation at 25°C. Its activation often followed that of the procollagenase present in the same media. 3. Activation of neutral proteinase (as does that of procollagenase) by trypsin or plasmin involved two successive steps: the activation of a latent endogenous activator present in the media followed by the activation of neutral proteinase itself by that activator. 4. The proteinase degrades cartilage proteoglycans, denatured collagen (Azocoll) and casein at neutral pH; it is inhibited by EDTA, cysteine or serum. Collagenase is not inhibited by casein or Azocoll and is less resistant to heat or to trypsin than is the proteinase. Partial separation of the two enzymes was achieved by gel filtration of the media but not by fractional $(\text{NH}_4)_2\text{SO}_4$ precipitation, by ion exchange or by affinity chromatography on Sepharose–collagen. These fractionations did not activate latent enzymes. 5. Trypsin activation decreases the molecular weight of both latent enzymes (60 000–70 000) by 20 000–30 000, as determined by gel filtration of media after removal of heparin. 6. The latency of both enzymes could be due either to a zymogen or to an enzyme–inhibitor complex. A thermostable inhibitor of both enzymes was found in some media. However, combinations of either enzyme with that inhibitor were not re-activated by trypsin, indicating that this inhibitor is unlikely to be the cause of the latency.

Like several other connective tissues, bone and cartilage are subject to remodelling processes that involve in their destructive phase the lysis of the insoluble fibres and macromolecules of an extracellular matrix, mainly collagen and proteoglycans. The cellular secretion of hydrolytic enzymes is presumed to play a critical role in these phenomena. Evidence for the participation of lysosomal acid hydrolases in resorption of bone (Vaes, 1968, 1969) or cartilage (Fell & Dingle, 1963; Dingle, 1975; Barrett, 1975; Poole, 1975) has been presented, but it is uncertain whether these enzymes are active in the first extracellular attack on the matrix. Their role could possibly be limited to the intracellular digestion of fragments freed from the matrix under the extracellular action of other hydrolases. Proteinases active at neutral pH have been suspected to initiate the sequence of degradative processes. The participation of a neutral collagenase (EC 3.4.24.7) (for a review see Harris & Cartwright, 1977) in the degradation of the collagen fibres of the matrix of bone or cartilage has to be

considered, and other neutral proteinases could possibly initiate the degradation of the proteoglycans.

We have shown (Vaes, 1972a) the presence, in the media surrounding mouse bone explants in culture, of a latent collagenase (or ‘procollagenase’) that could be activated by limited proteolysis obtained as a routine by trypsin treatment. Vaes (1972b) found that trypsin acted indirectly through the activation of a proteolytic enzyme (or ‘endogenous activator’) which is able to activate the procollagenase and which is also present in a latent form in the culture fluids. Moreover, a neutral proteinase, active on casein, was detected in the same culture media, where it is also present in a latent trypsin-activatable form, and the possibility was raised that this neutral proteinase could be the endogenous activator of procollagenase (Vaes, 1972a,b; Vaes & Eeckhout, 1975).

We have now pursued these studies and we have established that the bone explants that release procollagenase also release in a closely parallel manner a distinct latent neutral proteinase active, not only on

casein, but also on the protein core of proteoglycans and on denatured collagen (Azocoll). In the present paper, we report this simultaneous release and discuss observations bearing on the nature, on the latency and on the possible activators of the neutral proteinase, and we compare these properties with those of procollagenase as they were established in our previous work (Vaes, 1972a,b; Eeckhout & Vaes, 1977). The mechanism of the latency (zymogen or enzyme-inhibitor complex) is also discussed. Part of the work has been presented as a preliminary note (Vaes *et al.*, 1976) and in the context of a general review of our work on procollagenase (Vaes & Eeckhout, 1975).

Experimental

Tissue culture of bone and handling of the culture media

Whole tibiae or, when indicated, calvaria from 5-day-old mice were cultured in serum-free Eagle's basal medium containing 300 µg of heparin/ml as described by Vaes (1972a). Unless otherwise specified, the culture fluids were harvested after 4 days. Collection and handling of the media and their activation by trypsin were performed as described by Vaes (1972a). Higher concentrations of trypsin (6 or 8 µg/ml) were, however, used as a rule to activate the latent proteinase; they were followed by the addition of higher concentrations of soya-bean trypsin inhibitor (at least 32 µg/ml) to block the activity of trypsin completely before the proteinase assays. 'Subactivated' media [i.e. culture media that have been in limited contact with exogenous activator (e.g. trypsin or plasmin) insufficient to elicit any significant collagenase or proteinase activities from their precursors] were prepared as previously described (Vaes, 1972b; Eeckhout & Vaes, 1977).

Assays of neutral proteinase and of collagenase

Neutral proteinase activities were demonstrated and assayed on three different substrates: casein, Azocoll and cartilage proteoglycans.

The caseinolytic activity of the culture fluids was initially assayed by incubating together at 37°C for up to 4 h equal volumes of the enzyme preparation and of a 1% (w/v) solution of casein in 0.1 M-sodium cacodylate/HCl buffer, pH 7.0, containing 1 mM-CaCl₂. The trichloroacetic acid-soluble degradation products of casein were measured in the neutralized filtrate by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. One unit of activity refers to the amount of enzyme that releases per min trichloroacetic acid-soluble degradation products developing a colour density equivalent to that obtained with 1 µg of albumin. The assay mixture provided reaction products in amounts directly pro-

portional to the amount of enzyme preparation added up to 15% lysis (in albumin equivalent) of the substrate when incubated for up to 4 h; linearity with time was not observed after 2 h of incubation.

Greater sensitivity and precision were obtained by measuring the caseinolytic activity on [³H]acetylated casein. The assays were done by incubating together for 20–120 min at 37°C equal volumes of the [³H]-casein solution (9 mg of casein/ml; 300 000 d.p.m./mg of casein, prepared as described by Klimman & Karush, 1967) and of the enzyme preparation. The radioactivity of the trichloroacetic acid-soluble degradation products was measured in a liquid-scintillation counter after the addition of 10 vol. of scintillation liquid (Aqualuma). One unit of activity refers to the amount of enzyme that degrades 1 µg of casein into trichloroacetic acid-soluble fragments/min; this unit is arbitrary, as we assume that the specific radioactivity of the products soluble in trichloroacetic acid is the same as that of the substrate. The amount of acid-soluble radioactivity produced was directly proportional to the amount of enzyme added to the assay mixture up to 20% degradation of the original [³H]casein substrate when incubated for up to 120 min; linearity with time was not observed after 40 min of incubation.

The gelatinase activity of the culture fluids was assayed on Azocoll, an insoluble denatured collagen complexed with a red azo dye (Todd, 1949; Oakley *et al.*, 1946). The soluble red products released by proteolysis of the substrate were measured in the supernatant by their *A*₅₂₅; the complete proteolytic solubilization of the substrate (Azocoll, 20 mg) gave an *A*₅₂₅ of 6.8 under our working conditions. One unit of activity refers to the amount of enzyme that degrades 1 µg of insoluble Azocoll into soluble fragments/min. The *A*₅₂₅ of the soluble reaction products increased linearly with the amount of enzyme preparation added to the assays up to the hydrolysis of at least 10% of the substrate; linearity with time of incubation was observed for at least 80 min.

The proteolytic activity on cartilage proteoglycans was usually measured as described by Hauser & Vaes (1977, 1978) and Vaes *et al.* (1977) by the release of soluble ³⁵S-labelled material from discs of rabbit ear cartilage biosynthetically labelled with ³⁵S in their proteoglycan molecules. These discs were prepared as described by Ignarro *et al.* (1973). The cartilage cells were then killed by repeated freezing and thawing and the cartilage was washed extensively before the enzyme assays. To minimize autolysis of the proteoglycans during long-term incubations, the cartilage discs were usually heated for 30 min at 60°C before the assays; control experiments were done on non-heated cartilage discs to ensure that the proteinase was also active on native proteoglycans. All assays were done in triplicate or in quadruplicate. The amount of ³⁵S-labelled fragments released by the

enzyme from the cartilage proteoglycans was expressed as a percentage of the total amount of ^{35}S initially present in the assay. One unit of activity refers to the amount of enzyme that releases 1% of the ^{35}S radioactivity initially present in the cartilage/min.

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 in conditions under 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).

Collagenase assays were done on soluble native [^{14}C]collagen (20000–60000 d.p.m./mg) at 25°C (maximum incubation time 30 min) as previously reported (Vaes, 1972a). The results are expressed either as the radioactivity of the supernatant obtained after sedimentation (195000 g·min) of the residual collagen fibrils or in units/ml of culture fluid, one unit referring to the degradation of 1 μg of native soluble collagen/min at 25°C. We have established that this corresponds approximately to the degradation of 1 μg of reconstituted collagen fibrils/min at 37°C. In some experiments, the neutral proteinase active on Azocoll and collagenase was assayed at 25°C in the same incubation mixture.

Concentration and chromatographic fractionations of the culture media

Usually the culture media used for the fractionation studies were first concentrated by ultrafiltration under N_2 pressure on an Amicon Diaflo UM-10 or PM-30 membrane. For some experiments, however, and to eliminate the heparin present in the culture media (Sakamoto *et al.*, 1975), the proteinases present in the media were concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation by adding in the cold 6 vol. of satd. $(\text{NH}_4)_2\text{SO}_4$ solution to 4 vol. of media. The precipitate was collected by centrifugation (180000 g·min), washed with 2 × 10 vol. of satd. $(\text{NH}_4)_2\text{SO}_4$ and precipitated again by centrifugation as above. The washed precipitate was then dissolved in 5 ml of 0.05 M-sodium cacodylate/HCl buffer, pH 7, containing 0.15 M-NaCl and 5 mM- CaCl_2 and dialysed extensively against the same buffer.

Fractionation by $(\text{NH}_4)_2\text{SO}_4$ precipitation of the culture media was carried out at 0–4°C by addition of the appropriate volumes of satd. $(\text{NH}_4)_2\text{SO}_4$ solution to the media. Precipitates formed, in a first experiment at 0–20, 20–40 and 40–60% $(\text{NH}_4)_2\text{SO}_4$ saturation, and in a second experiment at 0–30, 30–40, 40–50 and 50–60% $(\text{NH}_4)_2\text{SO}_4$

saturation, were collected by centrifugation as above, dissolved in a small volume of 0.05 M-Tris/HCl buffer, pH 7.5 (at 25°C), containing 0.15 M-NaCl, 5 mM- CaCl_2 and 0.2 mg of NaN_3 /ml and dialysed extensively against that buffer. Both enzymes were completely precipitated from the media at 60% $(\text{NH}_4)_2\text{SO}_4$ saturation.

Gel filtration of concentrated culture media was done with calibrated columns of Sephadex G-200, Sephadex G-150 or Ultrogel Aca 44 as described by Vaes (1972a), by using either 0.15 M- or 1 M-NaCl in the eluting buffer. Fractionation by affinity chromatography on Sepharose–collagen was performed as described by Gillet *et al.* (1977). Ion-exchange chromatography was done at 4°C on columns (3 cm × 1.6 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with 10 mM-sodium cacodylate/HCl buffer, pH 7, containing 0.15 M-NaCl, 1 mM- CaCl_2 and 0.2 mg of NaN_3 /ml. Elution was done by increasing linearly (150 ml total vol.) the concentration of NaCl in the eluent up to 1 M; the flow rate was about 25 ml/h.

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 activated culture fluid as described above. A 0.5 ml sample of the digest, containing the ^{35}S -labelled soluble degradation products released from the cartilage, was filtered on a Sepharose 6B column (20 cm × 1.6 cm; V_0 = 10.6 ml; 25 ml/h; 1 ml fractions) equilibrated and eluted with 0.05 M-sodium cacodylate/HCl buffer, pH 7, containing 0.5 M-NaCl and 0.2 mg of NaN_3 /ml. The ^{35}S radioactivity was then measured (Hauser & Vaes, 1978) in the various fractions. Samples of cartilage digest were further degraded with papain (EC 3.4.22.2, 0.2 mg/ml) or chondroitinase ABC [EC 4.2.2.4, 2 units (as defined by suppliers)/ml] as described elsewhere (Hauser & Vaes, 1978) before being filtered on the same column.

Materials

Azocoll was obtained from Calbiochem (La Jolla, CA, U.S.A.), chondroitinase ABC from Sigma Chemical Co. (St Louis, MO, U.S.A.), papain (puriss) from Fluka A.G. (Buchs, Switzerland). $\text{Na}_2^{35}\text{SO}_4$, ^{35}S -labelled heparin and [^3H]acetic acid anhydride were from The Radiochemical Centre (Amersham, Bucks., U.K.), Ultrogel Aca 44 was from LKB (Stockholm, Sweden), Aqualuma was from Lumac S.A. (Meise, Belgium).

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

Fig. 1. *Comparative activation of neutral proteinase and of procollagenase by trypsin*

Culture medium was preincubated for the time indicated at 25°C with 2 µg of trypsin/ml before the addition of 80 µg of soya-bean trypsin inhibitor/ml. The results of two separate experiments are presented. The activation of collagenase (○) is compared with that of the neutral proteinase (●) assayed at 25°C on either casein for the experiment reported in (a) or on Azocoll (present together with collagen in the same incubation mixture) for the experiment reported in (b).

Results

Latency of neutral proteinase and its activation by trypsin or chymotrypsin

At pH 7.5, the culture media showed a proteolytic activity on a casein substrate, but only when they had first been activated by preincubation with trypsin or, less efficiently, with chymotrypsin (see Fig. 9 in Vaes & Eeckhout, 1975). The activation usually followed a sigmoidal time course, similar to that observed in the activation of collagenase by trypsin (Vaes, 1972a,b); an initial lag phase often preceded the activation proper (Fig. 1a). Trypsin activation of the culture media caused also the appearance of a significant proteolytic activity on Azocoll (Fig. 1b) and on cartilage proteoglycans (see below).

Parallel release of neutral proteinase and procollagenase during the cultures and effect of heparin

The bone explants released only insignificant amounts of either latent or active collagenase (Lenaers-Claeys & Vaes, 1976) or neutral proteinase (casein substrate) over 4 days' culture in a medium lacking heparin. The addition of increasing doses of heparin (12.5–600 µg/ml) to the culture fluid had no effect on the accumulation of the active enzymes, but it caused a progressive accumulation of both procollagenase and latent neutral proteinase in the media (Fig. 2), the maximum amount being obtained with 150–300 µg of heparin/ml. Under these conditions, the accumulation of both enzymes was closely parallel, a correlation coefficient $r=0.95$ being obtained between the activities measured for procollagenase and for neutral proteinase.

In the presence of heparin, neutral proteinase (casein or ^{35}S -labelled cartilage proteoglycan substrate) appeared in the culture media after a latency

Fig. 2. *Effect of heparin on the accumulation of neutral proteinase and of collagenase in the culture media*

Calvaria from 5-day-old mice were cultured for 4 days in the presence of increasing concentrations of heparin. Neutral proteinase ($[\text{H}^3]\text{casein}$ substrate) (●) and collagenase (○) were then assayed after trypsin activation of the culture media; no activity was detected in media that had not been first activated. Each point is the mean of five cultures; the vertical bars correspond to 1 S.D., shown arbitrarily in one direction only for the sake of clarity.

of 24–48 h and accumulated rapidly thereafter, reaching a maximum at day 4 or 5 of culture. Its accumulation closely paralleled that of procollagenase (Fig. 3), correlation coefficients r of 0.89 and 0.86 being obtained between the activities measured for procollagenase and, respectively, neutral protein-

Fig. 3. Time course of the accumulation of neutral proteinase, of collagenase and of their inhibitor in the culture media

Calvaria from 5-day-old mice were cultured for the time indicated in a medium containing $300\mu\text{g}$ of heparin/ml. In (b) collagenase ($\circ$) and neutral proteinase activities on either $[\text{H}]$ casein ($\bullet$) or ^{35}S -labelled cartilage proteoglycans (Δ) were then assayed in culture media that had first been activated by trypsin; no activity was detected in non-activated media. Each point is the mean of six cultures (12 cultures for day 3); the vertical bars correspond to 1 s.d., shown arbitrarily in one direction only for the sake of clarity. In (a) inhibitors of either collagenase ($\square$), or of neutral proteinase measured on $[\text{H}]$ casein ($\blacksquare$), were assayed in the pooled media of the same cultures by preincubating together 4 vol. of non-activated culture medium from these cultures with 1 vol. of an enzyme preparation containing both neutral proteinase and collagenase activities (auto-activated media from another culture). The results are expressed as percentage inhibition compared with controls incubated with non-cultivated culture medium.

ase assayed on $[\text{H}]$ casein or neutral proteinase assayed on ^{35}S -labelled cartilage. In this experiment, the calvaria also released inhibitors of either active neutral proteinase or collagenase; the inhibiting power of the culture media reached a maximum at days 3–5 and decreased thereafter. The inhibitors of neutral proteinase that are present in the culture media are further studied below.

To ascertain whether heparin had any direct effect on the latency of either neutral proteinase or colla-

genase, directly active and latent (trypsin-activatable) enzyme activities were measured on culture media that had been freed of heparin by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Six different culture media were tested, four contained only latent proteinase or collagenase and two contained a mixture of latent and of auto-activated (see below) enzymes. After the $(\text{NH}_4)_2\text{SO}_4$ precipitation, 56–99% of the initial enzyme activities were recovered in the sediment and no activity was left in the supernatant. Each precipitate contained the same ratio of active to latent enzyme activity as was initially present in the culture fluid before the $(\text{NH}_4)_2\text{SO}_4$ treatment. Thus, in confirmation of our previous experience (Vaes, 1972a) with procollagenase only, the latency of neither neutral proteinase nor collagenase appears to be the direct consequence of the presence of heparin in the culture medium. This was further checked for procollagenase by ion-exchange chromatography on DEAE-cellulose of media obtained from cultures grown in the presence of ^{35}S -labelled heparin ($1400\text{c.p.m.}/\mu\text{g}$). Although the ^{35}S -labelled heparin required an ionic strength of 0.5 to be eluted from the column, the collagenase was eluted in a completely latent form at an ionic strength of 0.3 in fractions devoid of ^{35}S .

Substrates and properties of activated neutral proteinase

The activated culture media exert a proteolytic activity on several substrates. They release soluble ^{35}S -labelled fragments from cartilage proteoglycans, soluble dyed products from insoluble Azocoll and trichloroacetic acid-soluble fragments from casein. The ^{35}S -labelled cartilage-degradation products released by the enzyme were eluted from 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 an exoglycosidase chondroitinase ABC (Fig. 4). Moreover, activated culture media decrease the specific viscosity of solutions of purified proteoglycan subunits obtained from bovine nasal cartilage (Fig. 5); however, these solutions had high residual specific viscosities that remained constant after a prolonged incubation.

The proteolytic activities on casein, Azocoll, or cartilage proteoglycan are all optimum at approx. pH 7–8 (Fig. 6). They are inhibited (Table 1) by EDTA (see also Fig. 5), *o*-phenanthroline, imidazole, cysteine, dithiothreitol and human serum, but not by di-isopropyl phosphorofluoridate, phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor, Trasylol, 4-hydroxymercuribenzoate, *N*-ethylmaleimide or heparin.

The caseinolytic enzyme obeyed Michaelis-Menten kinetics, with a K_m value of 1.3 mg of casein/ml. The rate of degradation of Azocoll was half-

Fig. 4. Gel filtration of the ^{35}S -labelled degradation products of cartilage on Sepharose 6B

The chromatographic fractionation of a 1mg solution of Blue Dextran (Δ) and of $\text{Na}_2^{35}\text{SO}_4$ (10000 d.p.m.) ($\circ$) in the eluent (a) was followed by chromatography of the soluble degradation products ($\bullet$) obtained after digestion of ^{35}S -labelled cartilage by activated culture media for 17h (b), 46h (c) or 90h (d). These digestions corresponded respectively to the release of 29, 90 and 94% of the total ^{35}S -label initially present in the cartilage. In experiment (c), chromatographs were done either on the 46h cartilage digest ($\bullet$) or on the digest that had been further degraded by papain ($\square$) or chondroitinase ABC (Δ). In experiment (d), fresh enzyme preparation (activated culture medium) was added at 66h to the cartilage digest to ensure that it was completely digested.

maximal for a concentration of 15.1 mg of Azocoll/ml. As shown in Table 1, casein inhibited the proteolytic activity on Azocoll, ^{35}S -labelled cartilage proteoglycans or proteoglycan subunits from bovine nasal cartilage (Fig. 5); it had, however, no effect on the activity of collagenase. Native salt-soluble collagen, at a concentration (0.5 mg/ml) corresponding to the K_m of collagenase (Vaes, 1972a), had no effect on the proteolysis of Azocoll, nor did the addition of Azocoll or of gelatin influence the activity of collagenase (Table 1).

Comparative activation of neutral proteinase and of procollagenase

Neutral proteinase can be activated together with procollagenase, by either kallikrein or plasmin

(Fig. 7). Dialysis of culture medium for 24h at 0°C against 3M-sodium thiocyanate, which Eeckhout & Vaes (1977) used to activate procollagenase, activated the neutral proteinase to the same extent as procollagenase, whereas dialysis against 3M-NaCl had no effect. Moreover, some untreated culture fluids activated themselves spontaneously if they were incubated for long periods at 25°C . As observed for procollagenase (Eeckhout & Vaes, 1977) the neutral proteinase of most culture fluids was not activated spontaneously, and when it was, the rate of this 'autoactivation' varied extensively from one culture medium to the other. An excellent correlation ($r=0.91$) was observed between the extent of autoactivation of neutral proteinase and of procollagenase whenever such autoactivation did occur.

As with the activation of procollagenase (Vaes, 1972b; Eeckhout & Vaes, 1977), the activation of neutral proteinase by trypsin appears to involve at least two steps: the activation of an endogenous latent activator followed by the activation of neutral proteinase itself. Indeed both the procollagenase and the neutral proteinase, assayed either on Azocoll (Fig. 8a) or on casein (Fig. 8b), were activated spontaneously in media 'subactivated' (see the Experimental section) by trypsin (Fig. 8a) or plasmin (Fig. 8b), when these media were further preincubated at 25°C after the inhibition of these enzymes. Thus a limited action of trypsin or plasmin on the culture fluids, which was insufficient by itself to elicit any free collagenase or proteinase activity from their latent precursors, nevertheless triggered an endogenous mechanism leading to their activation after a latency period of variable length. Further, as observed for procollagenase (Vaes, 1972b), trypsin-activated culture medium activated rapidly the latent proteinase present in subactivated media, whereas non-activated medium inhibited the spontaneous activation of that proteinase (Fig. 8b). This indicates that the activated medium contained an activator of both latent neutral proteinase and procollagenase, which was elicited by trypsin from an inactive precursor and which is able to activate the latent neutral proteinase and the procollagenase present in subactivated media. It suggests moreover that the non-activated medium contained an inhibitor of that endogenous activator.

It is important to observe that, in subactivated medium, the spontaneous activation of procollagenase precedes that of neutral proteinase (Fig. 8a). The same was observed when both enzymes were activated by trypsin and further incubated with their substrates under strictly comparable conditions (Fig. 1).

Gel filtration of culture media

Single peaks of either neutral proteinase or collagenase activity were recovered after gel fractionation

Fig. 5. Effect of non-activated and activated culture medium on the viscosity of a solution of proteoglycan subunits. In (a) 0.2 ml of either non-activated (○) or trypsin-activated (●) culture fluid was incubated at 37°C in Ostwald viscometers in a total volume of 0.7 ml containing 5 mg of purified proteoglycan subunits (from bovine nasal cartilage) in 0.1 M-Tris/HCl buffer (pH 7.4)/0.05 M-NaCl. The specific viscosity (η_{sp}) was calculated from the flow times measured at the times indicated. In another experiment (b), 0.2 ml of 3.5-fold-concentrated autoactivated (see the text) culture fluid was incubated under exactly the same conditions (●) or together with 14 mM-EDTA (□) or with 2 mg of casein/ml (Δ).

Fig. 6. Influence of pH on the proteinase activities of activated culture medium

Incubations were done in the presence of casein (a), Azocoll (b) or ³⁵S-labelled cartilage discs (c) in the following 0.1 M buffers: sodium acetate/acetic acid (Δ), sodium cacodylate/HCl (●), Tris/HCl (▲), glycylglycine/NaOH (○) and imidazole/HCl (□). This last buffer was a strong inhibitor of the neutral proteinase. In (c) — and — — — refer to results obtained in two independent experiments.

on Sephadex G-150 or G-200 of either non-activated (Fig. 9a) or activated culture media (Fig. 9b). The neutral proteinase peak contained all the proteolytic activities exerted on casein, Azocoll or ³⁵S-labelled cartilage proteoglycans. This gel filtration did not activate the latent neutral proteinase from non-activated culture media, even when the fractions containing the latent enzyme were pooled, re-concentrated by ultrafiltration (on Amicon PM 30) and passed again through a Sephadex G-200 column. The elution of either latent or activated neutral proteinase was slightly retarded compared with that of latent or activated collagenase respectively. By comparison with the results obtained by calibration of the columns, this behaviour corresponded to a difference of 21 000–23 000 between the apparent molecular weights ($\pm$ s.d.) of procollagenase ($137\,000 \pm 2\,000$; $n=6$) or collagenase ($104\,000 \pm 4\,000$; $n=4$) and those respectively of latent ($114\,000 \pm 11\,000$;

$n=6$) and active ($83\,000 \pm 14\,000$; $n=4$) neutral proteinase; by Student's *t* test, this difference is significant at $P<0.001$ for the latent and at $P<0.05$ for the activated enzymes. Moreover, the appearance of the activated neutral proteinase in the effluent was itself slightly retarded compared with that of the latent neutral proteinase, and the same difference was found between the elution of activated and latent collagenase. A mean difference of 31 000 was found between the apparent molecular weights of latent and activated neutral proteinase ($P<0.005$), a value very close to the mean difference of 33 000 ($P<0.001$) observed between the apparent molecular weights of latent and activated collagenase in these experiments.

However, when the gel filtrations on Sephadex G-150 were done in the presence of 1 M-NaCl (instead of 0.15 M-NaCl), lower apparent molecular weights were obtained for either latent ($78\,000 \pm 6\,000$; $n=3$) or activated ($58\,000 \pm 4\,000$; $n=4$) collagenase.

Table 1. *Effect of various agents on bone neutral proteinase and collagenase activities*

The agents of group (a) were preincubated for 15–30 min at pH 7.5 and 25°C together with the enzyme preparation (trypsin-activated culture medium) before the assays, whereas those of group (b) were added directly to the assays.

		Inhibition (%)			
		Proteolysis of			
Compound	Concn.	Casein	Azocoll	³⁵ S-labelled cartilage	Collagenase
Group (a)					
EDTA	15 mM	100	100	100	100
<i>o</i> -Phenanthroline	1 mM	96	—	100	100
Imidazole	50 mM	91	—	—	85
Di-isopropyl phosphorofluoridate	4 mM	—	—	0	—
	2 mM	0	—	—	0
Phenylmethanesulphonyl fluoride	2 mM	0	0	—	0
Soya-bean trypsin inhibitor	0.8 mg/ml	0	—	—	0
	0.15 mg/ml	0	—	0	—
Trasylol	1 mg/ml	0	0	—	0
Cysteine	10 mM	—	—	69	—
	2 mM	80	—	—	91
Dithiothreitol	1 mM	41	50	—	86
4-Hydroxymercuribenzoate	0.2 mM	0	—	—	0
<i>N</i> -Ethylmaleimide	10 mM	0	—	—	0
	4 mM	—	0	—	—
Heparin	0.6 mg/ml	0	—	—	0
Group (b)					
Human serum	10% (v/v)	—	—	100	—
	5%	100	—	—	85
	3.3%	87	—	—	81
	1%	39	—	—	31
Casein	4 mg/ml	—	—	100	0
	2.5 mg/ml	—	58	—	0
Azocoll	15 mg/ml	—	—	—	0*
Gelatin	3.75 mg/ml	—	—	—	0†
Collagen (soluble, native)	0.5 mg/ml	—	0‡	—	—

* Collagenase assayed with 0.2, 0.4 or 0.8 mg of collagen/ml.

† Collagenase assayed with 1 mg of collagen/ml.

‡ Proteolysis assayed with 5, 15, 25 or 35 mg of Azocoll/ml.

The elution behaviour of neutral proteinase (assayed on either casein, Azocoll or ³⁵S-labelled cartilage proteoglycans) was then similar to that of collagenase. However, gel filtrations of non-activated culture media under these conditions did not activate the latent collagenase or the neutral proteinase. Moreover, a difference of about 20000 ($P < 0.005$ by Student's *t* test) persisted between the molecular weights of latent and activated enzymes. Gel filtration of enzyme precipitated by 60%-satd. (NH₄)₂SO₄, on a column of Ultrogel AcA 44 in the presence of 0.15 M-NaCl, provided molecular-weight values for either latent (62000) or activated (38000) collagenase that were identical with those obtained for neutral proteinase. These values were also similar to those observed after gel filtration on the same column, but in 1 M-NaCl, of heparin-containing medium concentrated only by ultrafiltration (latent enzymes, 58000; activated enzymes, 39000). However, a difference of

about 20000 persisted again between the apparent molecular weights of latent and active enzymes.

Attempts to separate the neutral proteinase from the collagenase of the culture media

Gel filtration (see above and Fig. 9) did not allow complete separation of the neutral proteinase from the collagenase of the culture media. Fractionation by (NH₄)₂SO₄ precipitation also provided fractions [precipitated at 20–40, 30–40, 40–50, 40–60 or 50–60% (NH₄)₂SO₄ saturation] that contained both enzyme activities. Chromatography on ion-exchange columns of either activated or non-activated media (crude culture fluids or pooled reconcentrated fractions containing the enzyme obtained after gel filtration on Sephadex G-200) did not allow reproducible complete separation. Neither enzyme was adsorbed on columns of Bio-Rex 70, a cation-exchange resin, equilibrated with 10 mM-sodium cacodylate buffer, pH

Fig. 7. Activation of neutral proteinase and of procollagenase by plasmin and kallikrein

Neutral proteinase (closed symbols, assayed on [3 H]casein) and collagenase (open symbols) were assayed in culture fluids that had first been preincubated at 25°C in 40 mM-Tris/HCl buffer, pH 7.5, with either plasmin or kallikrein. In experiment (a), the preincubation was done for the indicated times, in the presence of 25 (Δ , $\blacktriangle$), 50 ($\square$, $\blacksquare$) or 100 ($\circ$, $\bullet$) µg of human plasmin/ml before the addition of 1 mg of soya-bean trypsin inhibitor/ml required to block plasmin action. In the controls ($\diamond$, $\blacklozenge$), soya-bean trypsin inhibitor (1 mg/ml) was first added to the culture medium before the addition of plasmin (100 µg/ml). In experiment (b), the preincubation was done for the indicated time in the presence of 700 µg of pig pancreatic kallikrein/ml ($\circ$, $\bullet$) before the addition of its inhibitor, di-isopropyl phosphorofluoridate (1.5 mM). In the controls ($\diamond$, $\blacklozenge$), the inhibitor was added before kallikrein to the culture medium.

Fig. 8. Activation of latent neutral proteinase and of procollagenase in 'subactivated' culture medium and evidence for an endogenous activator

(a) Non-activated culture medium ($\blacksquare$, $\square$) or 'subactivated' medium ($\bullet$, $\circ$), treated for 4 min at 25°C with 2 µg of trypsin/ml before the addition of 80 µg of soya-bean trypsin inhibitor/ml, was further preincubated at 25°C for the time indicated. Neutral proteinase ($\blacksquare$, $\bullet$) (with Azocoll substrate) and collagenase ($\square$, $\circ$) were assayed at 25°C in the same incubation mixtures. (b) 'Subactivated' medium (treated for 60 min at 25°C with 33 µg of plasmin/ml before the addition of 600 µg of soya-bean trypsin inhibitor/ml) was further preincubated at 25°C for the time indicated ($\bullet$) or after the addition of 0.05 vol. of medium fully activated by trypsin ($\blacktriangle$) or of 0.05 vol. of non-activated medium (Δ). Neutral proteinase was then assayed (on a [3 H]casein substrate).

5.5, containing 0.1 M-NaCl and 1 mM-CaCl₂, but they were largely retained on DEAE-cellulose at pH 7.0. A greater amount of collagenase than of neutral proteinase (assayed with casein or Azocoll as substrate) was then usually retained, which sometimes allowed recovery of either excluded fractions containing neutral proteinase without collagenase activity, or fractions eluted by the NaCl gradient containing collagenase without neutral proteinase. The yields were, however, low, owing to the inactivation of the enzymes during the purification procedures. Colla-

genase was also usually purified together with neutral proteinase (casein or Azocoll substrate) by affinity chromatography on Sepharose-collagen (Gillet *et al.*, 1977). In several cases, however, a peak of material devoid of collagenase and containing up to 20% of the neutral proteinase activity was eluted from the columns at 0.15 M-NaCl, but collagenase preparations free of neutral proteinase could not be obtained despite the high specific activity achieved for collagenase by this method (3500 units of latent collagenase/mg of protein: see Gillet *et al.*, 1977).

Fig. 9. Gel filtration of culture media on Sephadex G-150. ●, Neutral proteinase activity (with casein as substrate); ○, collagenase; ▲, A_{280} (protein). The void volume of the column was 59 ml. (a) Non-activated media. Neutral proteinase and collagenase assays were done after trypsin activation of the fractions; no directly measurable activity was found in the fractions for either enzyme when they were not activated by trypsin. (b) Trypsin-activated media.

A relative enrichment of the culture media in neutral proteinase activity compared with collagenase activity could also be obtained owing to a greater resistance of the neutral proteinase to denaturation. For instance, heat-inactivation of culture medium (10 min at 60°C) caused a 91% loss of procollagenase, but only a 58% loss of latent neutral proteinase (Azocoll or casein substrate). Similarly, preincubation of culture medium for 10 min with an excess of trypsin (80 µg/ml) caused a 95% loss of collagenase but only a 61% loss of neutral proteinase (casein substrate).

Inhibitors of neutral proteinase in the culture media

Several attempts to demonstrate the presence of an inhibitor of neutral proteinase (casein substrate) in the media surrounding cultured tibiae or in the fractions obtained after gel filtration of these media on Sephadex G-200 were unsuccessful. These attempts were done by preincubating before the assays 1–3 vol. of non-activated media or fractions with trypsin-activated media. However, an inhibitor was found in the fluids surrounding cultured calvaria. It was

effective both on the neutral proteinase and on the collagenase of either autoactivated or trypsin-activated culture media, a complete inhibition being sometimes obtained (as shown at days 4–5 on Fig. 3) by preincubating 4 vol. of non-activated medium with 1 vol. of active medium. The inhibitor was heat-stable and it resisted trypsin treatments that activate latent neutral proteinase and procollagenase (Table 2). Of particular importance with regard to the possible role of this inhibitor as a cause of the latency of either neutral proteinase or collagenase is the observation that combinations of autoactivated enzyme (either neutral proteinase or collagenase) with that inhibitor were not re-activated when they were incubated with an excess of trypsin (Table 3).

Discussion

The proteinase found in a latent state in the culture fluids surrounding mouse bone explants in the present experiments is active on cartilage proteoglycans, on denatured collagen (Azocoll) and on casein. The first activity is best interpreted as resulting from the lysis of the protein core of the proteoglycan macromolecule. Indeed, the soluble ^{35}S -labelled products obtained from ^{35}S -labelled cartilage discs under the action of the enzyme are further digested into smaller pieces by papain, a proteinase known to release from cartilage proteoglycans peptide fragments which contain mainly a single glycosaminoglycan chain (Luscombe & Phelps, 1967; Mathews, 1971; Heinegård & Hascall, 1974), and can thus not be fragments of polysaccharide chains released by a glycosidase. Moreover, as the enzyme also lowers the viscosity of solutions of purified proteoglycan subunits, these soluble products should be considered as fragments obtained by the proteolytic degradation of these subunits.

The proteolytic activity exerted on the three substrates is optimum at approx. pH 7 and is inhibited by metal-binding agents, but not by inhibitors of serine proteinases nor by thiol-blocking agents. It is thus likely to be due to a neutral metal-dependent proteinase. Proteoglycan-degrading neutral metalloproteinases similar in several respects to the present one have been found in directly active form in human cartilage tissue (Sapolsky *et al.*, 1976) or as a component of the secretion of rabbit bone-marrow macrophages or fibroblasts in culture (Hauser & Vaes, 1977, 1978; Vaes *et al.*, 1977; G. Huybrechts-Godin & G. Vaes, unpublished work). These enzymes are different from the two proteoglycan-degrading neutral serine proteinases (elastase and the 'chymotrypsin-like' enzyme, cathepsin G) present in human polymorphonuclear leucocytes (for a review, see Janoff, 1975). It is, however, uncertain whether the proteolytic activities exerted by the bone culture media on casein, Azocoll and proteoglycans are due to a single enzyme or to

Table 2. *Inhibition of neutral proteinase and of collagenase by an inhibitor present in the culture medium of explanted calvaria*

In Expt. A, samples (1 ml) of an autoactivated culture medium (1–6) or of fresh non-cultivated medium (7) were preincubated for 15 min at 25°C with 4 ml of fresh non-cultivated medium (1) or of calvaria culture medium (obtained after 4 days culture). The calvaria medium was used as such (2) or after heat-treatment for 10 min at 100°C (3) followed by digestion with trypsin (10 µg/ml) for 1 (4), 5 (5) or 10 (6) min; in medium (7), it was first activated by trypsin (10 µg/ml) for 10 min. Whenever trypsin was used, its activity was fully blocked by the addition of an excess of soya-bean trypsin inhibitor before the preincubations. Neutral proteinase ($[^3\text{H}]$ casein substrate) and collagenase were then assayed in these mixtures. In Expt. B, samples (1 ml) of trypsin-activated (10 µg/ml for 10 min) culture medium were preincubated for 15 min at 25°C with 4 ml of fresh non-cultivated medium (1) or of calvaria culture medium that had been heated (2) and digested for 1 (3), 5 (4) or 10 (5) min with trypsin as described in Expt. A. Neutral proteinase and collagenase were then assayed in these mixtures.

Preincubated mixtures of culture media (1 ml+4 ml)	Neutral proteinase		Collagenase	
	Units	Units inhibited	Units	Units inhibited
Expt. A				
(1) Autoactivated+fresh	1.1	—	5.2	—
(2) Autoactivated+calvaria	0.2	0.9	1.0	4.2
(3) Autoactivated+calvaria, heated	0	1.1	0	5.2
(4) Autoactivated+calvaria, heated, trypsin (1 min)	0	1.1	0	5.2
(5) Autoactivated+calvaria, heated, trypsin (5 min)	0.1	1.0	0	5.2
(6) Autoactivated+calvaria, heated, trypsin (10 min)	0	1.1	0	5.2
(7) Fresh+calvaria, trypsin (10 min)	7.4	—	14	—
Expt. B				
(1) Trypsin-activated+fresh	5.6	—	11.5	—
(2) Trypsin-activated+calvaria, heated	3.3	2.3	6.9	4.6
(3) Trypsin-activated+calvaria, heated, trypsin (1 min)	3.8	1.8	7.1	4.4
(4) Trypsin-activated+calvaria, heated, trypsin (5 min)	4.4	1.2	7.8	3.7
(5) Trypsin-activated+calvaria, heated, trypsin (10 min)	3.7	1.9	7.7	3.8

Table 3. *Attempt to re-activate with trypsin combinations of autoactivated neutral proteinase or collagenase with their inhibitor*

Autoactivated culture medium (Expts. 1 and 2) or fresh non-cultivated medium (Expts. 3 and 4) were mixed with 4 vol. of fresh non-cultivated medium (Expt. 1) or of calvaria culture medium (obtained after 4 days culture). The calvaria medium was used either as such (Expt. 3) or after heat-treatment for 10 min at 100°C (Expts. 2 and 4). The mixtures were preincubated for 15 min at 25°C. Neutral proteinase ($[^3\text{H}]$ casein substrate) and collagenase were then assayed in these mixtures, either directly or after their activation by trypsin (5 µg/ml for 10 min, followed by the addition of 200 µg of soya-bean trypsin inhibitor/ml).

Preincubated mixtures of culture media (1 vol.+4 vol.)	Neutral proteinase (units/ml)		Collagenase (units/ml)	
	Direct assay	After trypsin	Direct assay	After trypsin
(1) Autoactivated+fresh	2.5	3.0	6.8	6.3
(2) Autoactivated+calvaria, heated	0	0.25	0	0.6
(3) Fresh+calvaria	0.13	11.3	0	16.7
(4) Fresh+calvaria, heated	0.16	0	0	0

several proteinases. Several data favour the single-enzyme hypothesis. Indeed the activities on the three substrates have similar responses to potential inhibitors; all three are latent and are activated by the same agents; they remain associated during various fractionation procedures (gel filtration, ion-exchange chromatography, affinity chromatography on Sepharose-collagen). Moreover, casein inhibits the proteolysis of either insoluble Azocoll or cartilage proteoglycans, as would be expected for a single enzyme. If a single latent enzyme is involved, it would thus be different in its specificity from the latent

gelatin-specific proteinase found in human leucocytes by Sopata & Dancewicz (1974).

The bone neutral proteinase is, however, distinct from the bone neutral collagenase. Collagenase is indeed not inhibited by casein or Azocoll. Moreover the activation of procollagenase by trypsin or its spontaneous 'autoactivation' precedes that of neutral proteinase. Neutral proteinase is also more resistant than collagenase to heat denaturation or to digestion by trypsin. However, with most of the chromatographic fractionation procedures used, including affinity chromatography on Sepharose-collagen,

neutral proteinase could not be significantly separated from collagenase. The reasons for this close association remain to be investigated. It is only by gel filtration on Sephadex G-150 or G-200 columns equilibrated with buffered 0.15M-NaCl that a partial separation of the two activities has been observed; the elution of neutral proteinase was then slightly retarded compared with that of collagenase. This difference did, however, disappear with gel filtration in 1M-NaCl or with gel filtration in 0.15M-NaCl of enzymes concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Moreover, the absolute values obtained for the molecular weights of neutral proteinase or collagenase from gel-filtration experiments in 1M-NaCl were significantly lower than the values obtained from gel filtrations in 0.15M-NaCl, as were those obtained from gel filtration in 0.15M-NaCl of $(\text{NH}_4)_2\text{SO}_4$ precipitates of the crude culture media. This indicates that the neutral proteinase and the collagenase have a similar, low apparent molecular weight, but that they both form with some constituents of the culture fluids complexes that are different for each molecule and that are dissociated at high ionic strength. The heparin present in the fluids may be involved in the formation of these complexes. Indeed Sakamoto *et al.* (1975) have shown that heparin could bind to the bone collagenase and that these complexes were dissociated at 10.8 or after $(\text{NH}_4)_2\text{SO}_4$ precipitation. The high and somewhat variable apparent molecular weights observed in the presence of 0.15M-NaCl for bone collagenase and neutral proteinase in the present and in previous work (Vaes, 1971, 1972a) are thus overestimated. Sakamoto *et al.* (1972) reported for active bone collagenase precipitated by $(\text{NH}_4)_2\text{SO}_4$ an apparent mol. wt. of 41 000, a value close to those that we observe under similar conditions in the present study. It is also likely that the charge effects of heparin in the complexes affect the ion-exchange-chromatographic properties of the enzymes and may be responsible for the lack of separation obtained by this method. The rapid inactivation of the enzymes during these chromatographic procedures have, however, so far prevented further attempts to dissociate and to separate by this technique the two enzymes from media from which heparin had been removed.

To draw a parallel with the production and activation of neutral proteinase, earlier work with procollagenase (Vaes, 1971, 1972a,b; Eeckhout & Vaes, 1974, 1977; Vaes & Eeckhout, 1975) was here repeated and confirmed. Just as for collagenase, the proteinase was almost completely latent in the culture fluids surrounding the bone explants. Its activation is closely similar to that of procollagenase, as it can be achieved not only by trypsin and chymotrypsin but also by plasmin and kallikrein as well as by dialysis of the culture fluids against 3M-NaSCN at 4°C and, for some culture fluids, by prolonged preincubation at 25°C, causing an apparent auto-

activation. Moreover, the culture media contain in a latent form an 'endogenous' activator of neutral proteinase, which was activated by trypsin or by plasmin and was similar to the endogenous activator of procollagenase (Vaes, 1972b; Eeckhout & Vaes, 1977) known to be present in these media. Throughout the present study, very close correlations were observed between the activation of either latent neutral proteinase or procollagenase under a variety of conditions. However, the activation of procollagenase was often found to precede that of neutral proteinase, indicating that the neutral proteinase is unlikely to be the endogenous latent activator of procollagenase, as initially suspected (Vaes, 1972a,b; Vaes & Eeckhout, 1975). Although the endogenous activator is inhibited by casein (see Fig. 7 in Vaes & Eeckhout, 1975), it is activated by 4-hydroxymercuribenzoate and by *N*-ethylmaleimide and inhibited by di-isopropyl phosphorofluoridate and propan-2-ol (Eeckhout & Vaes, 1977), whereas neutral proteinase is not affected by these agents.

These data suggest that similar molecular mechanisms are involved in the latency and in the activation of latent neutral proteinase and of procollagenase. It is important to observe that both latent enzymes remain latent after gel filtration in 1M-NaCl or after $(\text{NH}_4)_2\text{SO}_4$ precipitation and that, whatever the true molecular weight of the active enzymes may be, the activation of the latent enzymes by trypsin lowers their apparent mol.wt. by 20000–30000 under all the conditions of gel filtration that were investigated. Like procollagenase (Vaes, 1971, 1972a; Vaes & Eeckhout, 1975), latent neutral proteinase could thus be either a zymogen that is activated by a limited proteolysis of its molecule, or an enzyme inhibitor complex formed between the proteinase and some protein inhibitor, which is dissociated after partial proteolysis by trypsin, plasmin or another proteolytic enzyme. The activation of latent neutral proteinase by dialysis of culture fluids against 3M-NaSCN could result from the activation of the latent endogenous activator present in the fluids, a mechanism that probably explains the parallel activation of procollagenase under these conditions (Eeckhout & Vaes, 1977; Vaes & Eeckhout, 1975). Also the slow spontaneous activation observed when some culture fluids are incubated at 25°C for 24 or 48 h may be due to the presence of a small constant amount of an unidentified activator in these fluids, as suspected for the parallel spontaneous activation of procollagenase (Eeckhout & Vaes, 1977; Birkedal-Hansen *et al.*, 1976a,b).

Our present evidence does not allow a definitive choice between the two mechanisms (zymogen or enzyme-inhibitor complex) proposed to explain the latency of the neutral proteinase and discussed in our previous papers (Vaes, 1972a; Vaes & Eeckhout, 1975; Eeckhout & Vaes, 1977). An inhibitor of neutral

proteinase and of collagenase was found in some culture fluids. This inhibitor is heat-stable and trypsin-resistant and is effective against either auto-activated or trypsin-activated enzymes. It is thus different from the inhibitor detected by Sellers *et al.* (1977) in the culture media surrounding rabbit bone explants. The time sequence of production of that inhibitor and of latent neutral proteinase or collagenase by the bone explants in culture do not apparently provide any clue about the possible role of the inhibitor in the latency of either enzyme. The observations that combinations of neutral proteinase or of collagenase with the inhibitor were not re-activated by trypsin suggest strongly that the latent enzymes found in the culture fluids are not complexes formed between active enzyme and the present inhibitor.

The release of latent neutral proteinase by the bone explants in culture is closely parallel to that of procollagenase. Significant accumulations of either directly active neutral proteinase or of active collagenase were never observed in the culture media in our experiments. The accumulation of latent neutral proteinase was significant only when heparin was present, a fact observed for the accumulation of procollagenase (Lenaers-Claeys & Vaes, 1976) and reminiscent of the experience of others (Shimizu *et al.*, 1969; Sakamoto *et al.*, 1973) for directly active collagenase. The mechanism of this effect is so far unexplained (Vaes, 1972a; Lenaers-Claeys & Vaes, 1976). The addition of increasing doses of heparin to the culture fluids caused a progressive accumulation of both enzymes in a closely parallel manner. Also the time sequence of the accumulation of latent neutral proteinase and of procollagenase in the media during the cultures was closely parallel, both enzymes appearing after a latency period of 24–48 h and accumulating rapidly thereafter.

The simultaneous secretion of both enzymes allows us to raise two hypotheses. It suggests first that both enzymes may participate in the same physiological process, presumably extracellular matrix degradation. Indeed collagenase may initiate the fragmentation of the collagen molecule, allowing the denaturation of the fragments (for a review, see Harris & Cartwright, 1977) and their further degradation by the neutral proteinase, as suggested by evidence gathered in other systems (Harper & Gross, 1970; Harris & Krane, 1972; Harris, 1972). Moreover, our experiments indicate that the same neutral proteinase that acts on denatured collagen, or a closely related proteinase, also degrades cartilage proteoglycans. Thus the two main components of the extracellular framework of the connective tissues can be degraded into soluble fragments by these enzymes at about neutral pH. The soluble fragments formed under their action could then possibly be endocytosed and further digested within the lysosomal system of the cells.

A second hypothesis that arises from these observations is that both procollagenase and latent neutral proteinase are secreted by the same type of cells and by the same cellular mechanisms. The nature of that cell is, however, unknown, as the bone explants contain many different cell types: osteoblasts, osteocytes, osteoclasts, fibroblasts, cartilage cells etc. It is noteworthy that the secretion rate of these enzymes is different from that of lysosomal enzymes (β -glucuronidase, *N*-acetyl- β -glucosaminidase) as well as from the release of alkaline phosphatase or of lactate dehydrogenase (Lenaers-Claeys & Vaes, 1976). The various proteolytic enzymes active at neutral pH (e.g. collagenase, neutral proteinase) may thus possibly be part of a special secretory granule and be secreted together either as zymogens or as enzyme-inhibitor complexes to be activated outside the secreting cell. This would protect the cell against any proteolytic attack by its own product of synthesis and leave the control of the extracellular proteolytic activity to the balanced action of extracellular activator enzymes and of inhibitors. The fact that these precursors are apparently not stored in significant amounts inside the cells (G. Vaes & G. Lenaers-Claeys, unpublished work) may indicate another protective mechanism, consisting of the rapid extracellular release of the contents of the granules as soon as they have been synthesized.

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