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The Release of Collagenase as an Inactive Proenzyme by Bone Explants in Culture

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1. A latent collagenase, activated only by limited proteolysis, was found in culture media of mouse bone explants. It could be activated by trypsin or, less efficiently, by chymotrypsin. Skin explants also released latent collagenase. 2. Bone collagenase attacks native collagen at about neutral pH when it is in solution, in reconstituted fibrils or in insoluble fibres, producing two fragments representing 75 and 25% of the molecule. It requires calcium and is inhibited by EDTA, cysteine or serum. 3. Latent collagenase is not activated by trypsin-activated collagenase but by a distinct unidentified thermolabile agent present in a latent trypsin-activatable state in the culture media, or by purified liver lysosomes between pH 5.5 and pH 7.4. Trypsin activation decreases the molecular weight of latent collagenase from 105000 to 84000 as determined by gel filtration. 5. The latency of collagenase is unlikely to be due to an enzyme–inhibitor complex. Although some culture media contain a collagenase inhibitor, its presence is not constant and its molecular weight (at least 120000) is not compatible with the decrease in molecular weight accompanying activation; also combinations of collagenase with inhibitor are not reactivated by trypsin. Moreover, the latency remains after gel filtration, or treatment by high dilution, exposure to pH values between 2.5 and 10, or high ionic strength, urea or detergent. 6. It is proposed that latent collagenase represents an inactive precursor of the enzyme, a 'procollagenase', and that the extracellular activity of collagenase is controlled by another protease that activates procollagenase by a limited proteolysis of its molecule.

Several physiological or pathological processes are accompanied by the degradation and by the resorption of native collagen fibres in tissues or organs such as bone, skin, uterus, joint cartilage etc. (Harkness, 1961; Woessner, 1968). However, although denatured collagen is readily degraded by various animal proteinases, native collagen has long been known to be attacked only by a collagenase of bacterial origin (Mandl, 1961). Tissue extracts did not appear to contain any 'true' collagenase activity, that is, an activity demonstrable at neutral pH values on a native substrate, although they were shown to contain a non-specific collagenolytic activity that appeared only in acidic conditions. The collagen was presumably easily denatured at the acid pH values and the activity was attributed to the action of acid cathepsins of lysosomal origin (Woessner, 1962, 1968). However, the production of a true collagenase by several animal tissues in culture was demonstrated by Gross and coworkers (Gross & Lapière, 1962; Lapière, 1964; Nagai *et al.*, 1966); the enzyme was found in the culture medium only, being, as a rule, not measurable in tissue extracts (for a review see Gross, 1970).

Such a collagenase has also been found in the fluid surrounding bone explants in culture (Fullmer & Lazarus, 1967, 1969; Shimizu *et al.*, 1969). Efforts to demonstrate the activity failed until it was found that considerable collagenase activity could be elicited by exposure to trypsin. In the present paper, I will report and discuss observations bearing on the nature, on the latency and on the possible activators of this bone enzyme. They provide strong evidence that the collagenase is released as an inactive proenzyme that is activated by a limited proteolysis. Part of the work has been presented as a preliminary note (Vaes, 1971).

Experimental

Tissue culture of bone and skin

Whole tibiae were aseptically taken from 5-day-old NMRI mice, then dissected free of any adhering muscle or connective tissue. They were washed extensively to remove serum in culture medium containing 200 units each of penicillin and streptomycin/ml, and introduced into 20ml plastic roller tubes containing 2ml of tissue-culture medium. Four tibiae were laid on the wall of each tube, 3cm from the bottom, where they usually adhered during the whole culture period; a gas mixture of O_2+CO_2 (95:5) was then bubbled for 1 min into the medium before closing the tubes (Shimizu *et al.*, 1969). In a few experiments,

calvaria (the bones from the cranial vault) from 19-day-old NMRI mouse embryos were put into culture (4 calvaria per tube) as described by Vaes (1968) and gassed with CO_2+air (5:95). The skin of the scalp of these embryos was sometimes also cultivated in the same way. Two chemically defined tissue culture media were used without added serum: either Dulbecco's modified Eagle's medium (Dulbecco & Freeman, 1959) or Eagle's basal medium (BME) (Eagle, 1955) prepared in mammalian Tyrode solution from stock concentrates of amino acids, vitamins and L-glutamine. The media were supplemented by penicillin and streptomycin (100 units of each/ml) and by heparin (50 U.S. Pharmacopeia units or 294 μg/ml) (Shimizu *et al.*, 1969).

The tubes were incubated at 37°C in an almost horizontal position on a roller-tubes drum (6 rev./h) for two or three successive periods of 4 days each; the culture media were renewed at the end of each period. The collected media were pooled and immediately buffered by addition of 1 M-tris–HCl buffer adjusted to pH 7.5 with HCl, to a final concentration of 50 mM. They were then filtered on 0.45 μm Millipore filters to remove any floating cells or cell debris and assayed immediately or after storage at −20°C, after appropriate dilution with 50 mM-tris–HCl buffer, pH 7.5, containing 1 mM-$CaCl_2$.

Activation of the culture media

This was done by preincubating the tris-buffered (pH 7.5) media at 25°C for 10 min in the presence of 2 μg of trypsin/ml. Then at least 8 μg of soya-bean trypsin inhibitor/ml was added and the culture was incubated for a further 10 min at 25°C to completely block the activity of trypsin before the collagenase assays. Concentrated culture media or media supplemented with exogenous protein required larger concentrations of trypsin or longer incubation times to be activated. A quantitative evaluation of the latent collagenase could be obtained from a series of assays with culture media that had been submitted to increasing degrees of trypsin attack, by extrapolation of the exponential inactivation curve to zero time (see below: Fig. 2*a*). Less precise comparative evaluations could be made by comparisons of curves showing the time-course of activation of collagenase in various culture media preincubated with the same concentration of trypsin.

Preparation of [^{14}C]collagen

Radioactively labelled collagen was purified from the skin of actively growing male guinea pigs of approx. 200 g body weight that had received 150 μCi of [$^{14}C_1$]glycine intraperitoneally 6 to 8 h before they were killed. Neutral salt-soluble collagen was extracted in 0.5 M-NaCl and purified by the method of Gross (1958); the final product, which had a specific radioactivity of 20000 to 30000 c.p.m./mg, according to the preparation, was freeze-dried and stored in a dessicator at −20°C until use. Solutions for assays were prepared by dissolving the collagen (2 mg/ml) in 0.149 M-potassium phosphate buffer, pH 7.6; the solution was dialysed against 0.4 M-NaCl and the insoluble residue eliminated by ultracentrifugation (Lapière, 1964).

Collagenase assays on radioactive soluble collagen

Collagenase activity was assayed on native trypsin-resistant [^{14}C]glycine-labelled collagen either (Nagai *et al.*, 1966) by the release of soluble radioactivity from reconstituted fibrils (gel assay) or by the prevention of gel formation observed when dispersed collagen molecules have been degraded (solution assay). Results are expressed as the radioactivity of the supernatant obtained after sedimentation of the residual collagen fibrils.

Reconstituted collagen fibrils were prepared for the gel assays by preincubation of 50 μl of collagen solution in polypropylene 550 μl microtubes at 37°C for 18–24 h. Just before incubation, the fibrils were dispersed with a stainless-steel rod and 50 μl of 0.1 M-tris–HCl buffer, pH 7.5, containing 2 mM-$CaCl_2$ was added, followed by 100 μl of the medium to be assayed. The tubes were incubated for up to 4 h at 37°C, then 50 μl of 0.4 M-EDTA, adjusted to pH 7.5 with NaOH, was added to block the activity of the collagenase. The tubes were then immediately centrifuged at 13000*g* for 15 min at room temperature and the radioactivity was measured in a 100 μl sample of the supernatant solution in a liquid-scintillation counter. Blank values were obtained by parallel incubations made with culture medium that had first been heated for 15 min in a boiling-water bath to inactivate the collagenase.

Assays on collagen in solution were done by incubation in polypropylene microtubes for up to 4 h at 25°C of a mixture of 50 μl of collagen solution, 10 μl of 375 mM-tris–HCl buffer, pH 7.5, containing 7.5 mM-$CaCl_2$ and 15 μl of water (or 10 μl of water plus 5 μl of 0.4 M-EDTA, pH 7.5, for the blanks) together with 25 μl of the culture medium to be assayed. At the end of the incubation period, 25 μl of 0.4 M-EDTA (0.32 M for the blanks), pH 7.5, containing NaN_3, 1 mg/ml, as a bacteriostatic agent, was added to block the collagenase activity and the tubes were further incubated at 37°C for 18–24 h to allow the precipitation of intact collagen molecules as fibrils (gel formation). The tubes were then centrifuged at 13000*g* for 15 min at room temperature and the radioactivity was measured in a 50 μl sample of the supernatant solution in a liquid-scintillation counter.

Solution assays were done in duplicate and gel assays in triplicate. The former gave lower blanks and

better reproducibility, and were used with a 1h incubation time unless otherwise stated. Both assays provided reaction products in amounts directly proportional to the volume of culture medium added, up to the lysis of 30–40% of the collagen substrate, when the incubations were done for up to 4h; linearity with time was not observed after the first hour of incubation (Fig. 1). Trypsin at high concentration (50μg/ml) had only a limited action on the collagen substrate, resulting in the rapid solubilization of 5 to 15% of its radioactivity, according to the preparation, with no further hydrolysis thereafter.

Collagenase assay on insoluble collagen fibres

Collagenase was also assayed by the release of soluble hydroxyproline from insoluble collagen fibres (either a commercial preparation of purified bovine achilles tendon or a semi-purified preparation of insoluble collagen from guinea-pig derma). This last preparation is the fraction that was left after repeated extraction of the guinea-pig derma with 0.5M-NaCl during the preparation of [^{14}C]collagen; it contained 72.2μg of hydroxyproline/mg and was thus approx. 54% pure collagen, as hydroxyproline represents about 13.4% of the weight of mammalian collagen (Neuman & Logan, 1950).

Samples (20mg) of finely minced, freeze-dried insoluble collagen in 550μl polypropylene microtubes were mixed with 0.2ml of 0.1M-tris–HCl buffer, pH7.5, containing 2mM-$CaCl_2$, and the assays were started by the addition of 0.2ml of culture medium; the mixtures were incubated for up to 6h at 37°C. Blanks were run in parallel, with culture medium that had first been inactivated by heating for 15min in a boiling-water bath. At the end of the incubations, the tubes were centrifuged at 13000*g* for 15min at room temperature and 100μl of the supernatant solution were used for hydroxyproline determinations by the method of Bergman & Loxley (1963), after hydrolysis in sealed tubes in 6M-HCl for 22h at 107°C.

Viscometry

To 1.5ml of collagen solution were added 0.3ml of 0.375M-tris–HCl buffer, pH7.5, containing 7.5mM-$CaCl_2$ and 0.45ml of water; this mixture was heated to 25°C and the incubation was started by the addition of 0.75ml of culture medium; for the blanks, heat-inactivated culture medium was used. Then 2.5ml samples of this mixture were quickly transferred by means of a long Pasteur pipette to miniature U-tube viscosimeters (BS/U/M; size M4) preheated at 25°C in an all-glass water bath. Flow times, f_x, were read at intervals for about 24h; dividing f_x by the flow time, f_s, obtained in the same viscosimeter with the incubation mixture without collagen, gave the relative viscosity, $\eta_{rel.}$, from which the specific viscosity $\eta_{sp.}(=\eta_{rel.}-1)$ was then obtained.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the products of reaction of enzyme and collagen in solution at 25°C was performed by Dr. Charles Lapière, Department of Dermatology, University of Liège (Belgium) by the method of Furthmayr & Timpl (1971) after extensive dialysis of the reaction mixture against 0.1M-acetic acid at 4°C followed by freeze-drying. The size of the molecular fragments obtained after enzyme digestion was evaluated by reference to calibration curves made for α_1- and for α_2-derivatives by using as standards the uncleaved α-chains and some of their CNBr peptides.

Gel filtration of culture media

Gel filtration of culture media was done at 2–3°C with columns (1.5cm×85cm) of Sephadex G-200 (or G-100 in some experiments) equilibrated with 50mM-tris–HCl buffer, pH7.6, containing 0.15M-NaCl, 2mM-$CaCl_2$ and 0.2mg of NaN_3/ml. The column was calibrated by the use of markers including horse heart cytochrome *c* (mol.wt. 12400), bovine serum albumin (mol.wt. 67000), rabbit γ-globulins (mol.wt. 165000) and ox liver catalase (mol.wt. 240000). Pooled culture media were concentrated 10 to 20 times by ultrafiltration (see below) and a sample of 1 or 2ml of concentrated media was applied on the column, directly ('non-activated' media) or after preliminary activation by trypsin ('activated' media); the conditions for optimum activation were first determined for each pool of concentrated media. The column was run at a flow rate of 4.5ml/h (6ml/h for the G-100 column); fractions of 2.5ml (3ml for the G-100 column) were collected. When non-activated media were filtered, fractions were assayed for latent collagenase activity by adding 5μg of trypsin/ml of incubation mixtures; suitable amounts of soya-bean trypsin inhibitor were then added to the EDTA solution used to stop the reaction. This rapid procedure provided results that were qualitatively similar to those obtained when the effluents had been activated by trypsin in a preliminary preincubation.

Ultrafiltration and ultradialysis of culture media

Culture fluids were concentrated by ultrafiltration under N_2 pressure in an Amicon model 202 ultrafiltration cell with Diaflo UM-10 membranes that retain molecules with a molecular weight above 10000. For some experiments, ultrafiltrations or

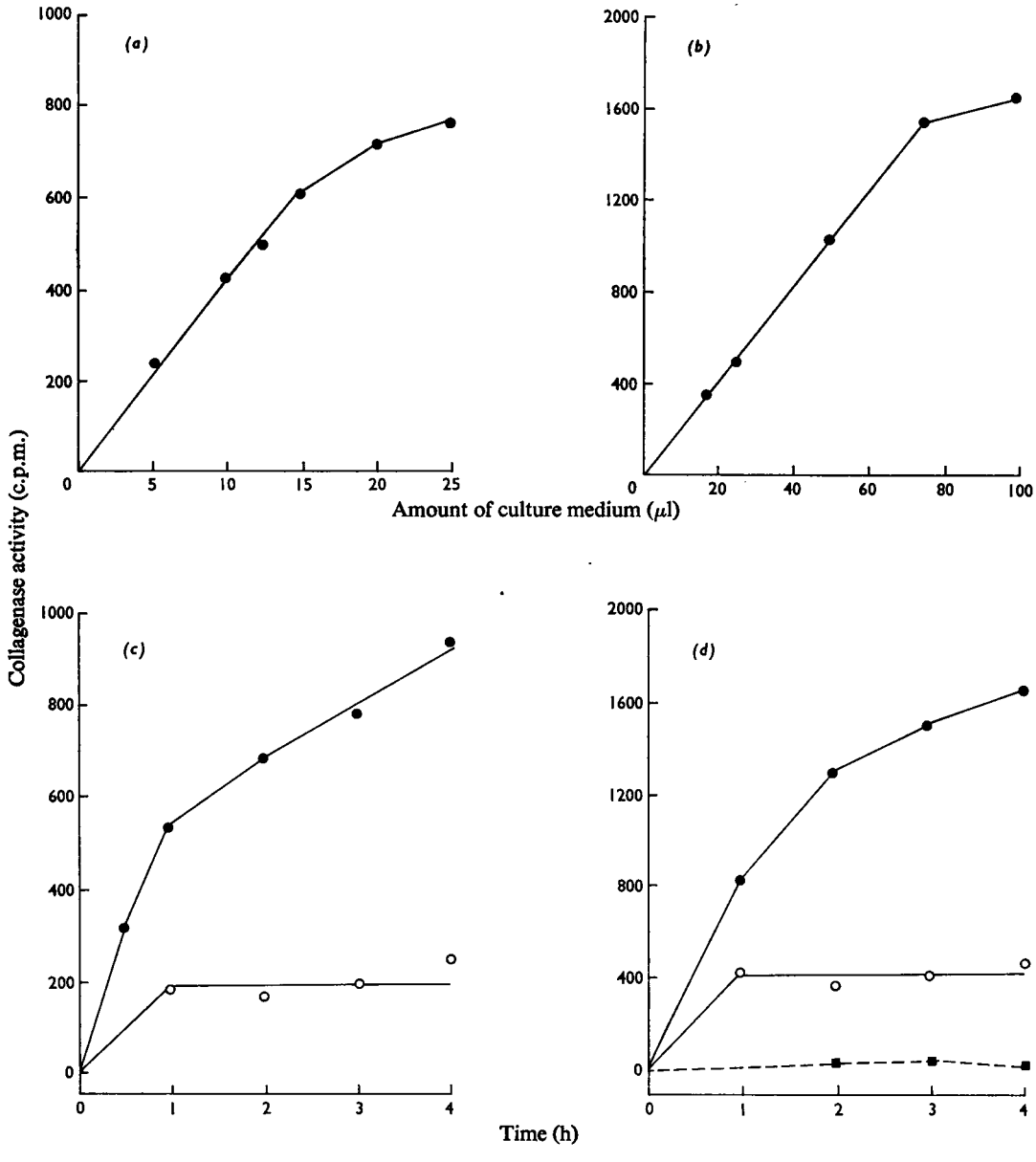

Fig. 1. *Kinetics of activated bone collagenase, non-activated bone collagenase and trypsin on collagen substrate in solution or in reconstituted fibrils*

The results of different experiments in which enzyme concentration (*a* and *b*) or incubation time (*c* and *d*) were varied, show the kinetics of activated (●) or non-activated (■) bone collagenase and of trypsin (○) on a collagen substrate in solution (*a* and *c*) or in reconstituted fibrils (*b* and *d*). The incubation time was 1 h for (*a*) and 4 h for (*b*). Trypsin, in 50 mM-tris–HCl buffer, pH 7.5, containing 1 mM-$CaCl_2$, was used at a final concentration of 50 μg/ml in Expts. *c* and *d*. There was approximately 2000 c.p.m. of substrate/tube for the solution assays (*a* and *c*) and 3000 c.p.m./tube for the gel assays (*b* and *d*).

ultradialysis were also done with PM-30 or XM-50 membranes, that retain molecules of mol.wt. approx. 30000 or 50000 respectively.

Preparation of purified liver lysosomes

Purified liver lysosomes were prepared by the method of Trouet (1969) from either rats or mice that had been injected with Triton WR-1339. The livers were frozen and thawed ten times (in a mixture of solid CO_2 and propan-2-ol) to rupture their membranes and to allow the action of the lysosomal enzymes on their substrates.

Materials

Dulbecco's modified Eagle's medium, basal Eagle's medium stock concentrates (100×) and penicillin–streptomycin mixtures (5000 units of each/ml) were obtained from Microbiological Associates, Bethesda,

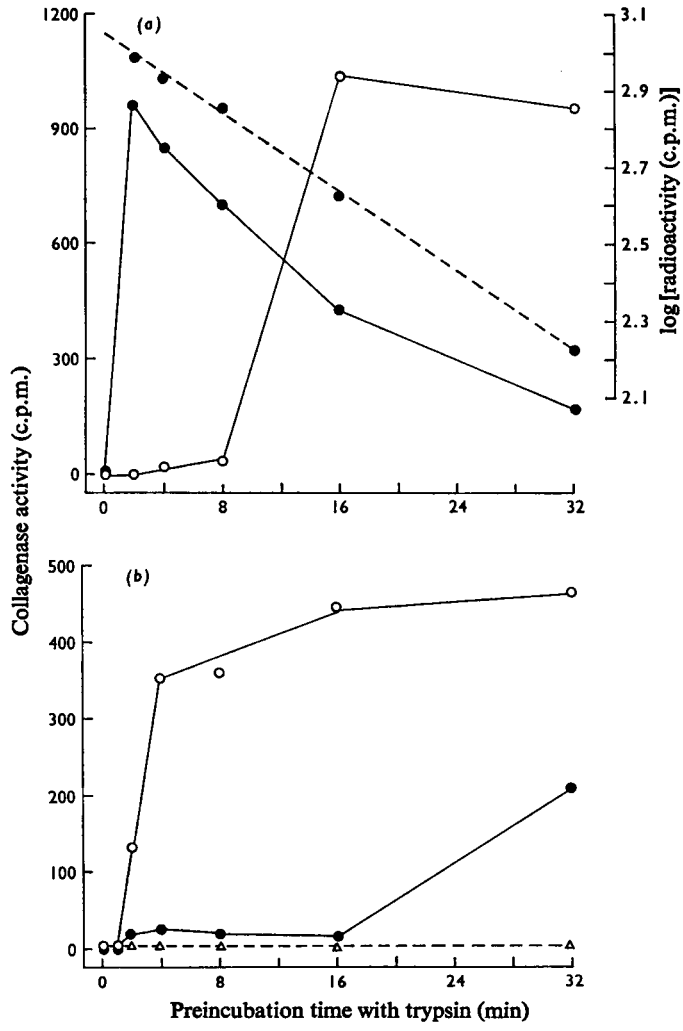

Fig. 2. *Time-course of the activation of bone collagenase by trypsin*

(*a*) Culture media concentrated by ultrafiltration were either activated by trypsin (10μg/ml) before being diluted for their further assay (○) or diluted before their activation by trypsin (●); --- refers to the scale on the right (log[radioactivity (c.p.m.)]) and shows the exponential inactivation course of the collagenase under excess-of-trypsin action. (*b*) Culture media were activated by trypsin (2μg/ml) either in the absence (○) or in the presence of 2.5mg (●) or of 10mg (△) of casein/ml.

Md., U.S.A. Heparin sodium salt, grade I, and collagen from bovine Achilles tendon were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Trypsin (2× or 3× crystallized), α-chymotrypsin and soya-bean trypsin inhibitor were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Casein (Hammersten quality) was from Nutritional Biochemicals, Cleveland, Ohio, U.S.A., and [$^{14}C_1$]glycine was from C.E.N., Mol, Belgium.

Results

Latency of collagenase and its activation by trypsin

Very low and inconsistent amounts of collagenase activity were observed when assays were done directly on the culture media of explanted tibiae. Considerable activity became evident when the media had been submitted to a limited proteolysis, either by adding trypsin to the incubation mixtures, or by preincubation with trypsin then soya-bean trypsin inhibitor. The greatest amounts of activity were found in the media collected after the first 4-day period of culture, but almost no activity was found after day 8. Latent collagenase activity was also found in the media from embryonic calvaria cultivated under the same conditions, as well as in culture media from embryonic skin explants; with skin, no activity was found in the first media (days 1–4) but weak latent collagenase was evident in the second (days 5–8).

Activation of collagenase by trypsin usually followed a sigmoidal course; an initial lag phase often preceded the activation proper (Fig. 2). This lag period was longer when concentrated culture fluids were assayed (Fig. 2*a*) or when the media had been supplemented with an exogenous protein, such as casein, that does not compete with the action of collagenase on collagen (Fig. 2*b*), suggesting that it was at least partly due to the presence of proteins that compete for trypsin with the latent collagenase. With excess of trypsin the activity decreased exponentially (Fig. 2*a*), suggesting that proteolytic destruction of the collagenase was occurring.

Chymotrypsin could also activate collagenase but it was 20–40 times less effective than trypsin (Fig. 3); the same difference was observed when the activation was done on a latent collagenase preparation that had been partially purified by gel filtration.

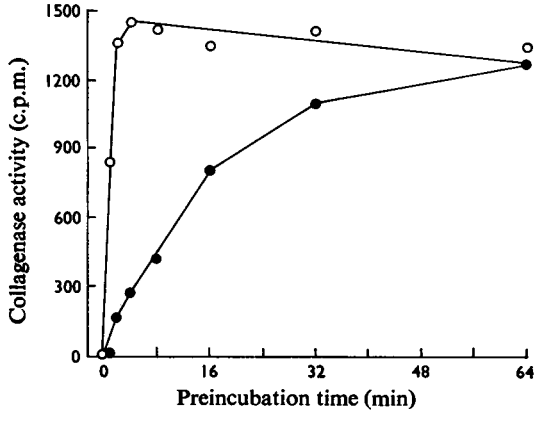

Fig. 3. *Activation of collagenase by chymotrypsin and by trypsin*

Culture medium was preincubated with 2 μg of trypsin/ml (○) or with 5 μg of chymotrypsin/ml (●) before the addition of the 200 μg of soya-bean inhibitor/ml required to inhibit chymotrypsin as well as trypsin.

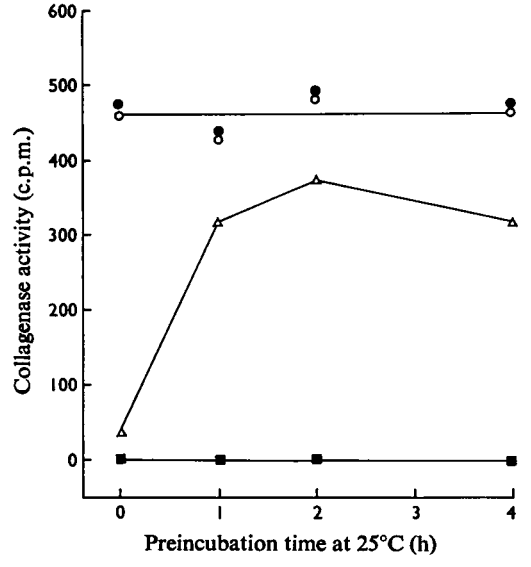

Fig. 4. *Activation of latent collagenase in 'sub-activated' culture medium*

Non-activated culture medium (■), or medium fully activated by treatment for 10 min at 25°C with 2 μg of trypsin/ml (○), or 'sub-activated' medium, treated for 1 min only at 25°C with 2 μg of trypsin/ml (Δ), were diluted by half with 50 mM-tris–HCl buffer, pH 7.5, containing 1 mM-$CaCl_2$; non-activated medium was also mixed at equal volumes with fully activated medium (●). Collagenase was assayed in these mixtures either directly or after their preincubation at 25°C for various lengths of time. There was no difference between the results obtained with buffer-diluted non-activated medium (■) and with the same dilution of non-activated medium that had been added to a mixture of trypsin and excess of soya-bean trypsin inhibitor.

Chymotrypsin activation is unlikely to be due to a trypsin contaminant as it persisted in the presence of small concentrations of soya-bean trypsin inhibitor.

Activation by an agent present in the culture medium

Up to 4h of preincubation at 25°C of non-activated medium with the same medium that had been first fully activated by trypsin did not result in the activation of the latent collagenase present in the non-activated medium (Fig. 4). However, 'sub-activated' medium, that is medium which had been in a limited contact with trypsin, insufficient for a full activation of collagenase (the activation by trypsin had been stopped by the addition of trypsin soya-bean inhibitor at some time during the lag period preceding the activation proper), activated itself during a further preincubation at 25°C in the absence of substrate (Fig. 4). Activation of latent collagenase in 'sub-activated' medium was also obtained at 0°C by the action of medium fully activated by trypsin; the activating factor was then found to be distinct from the activated collagenase, as the latter was more rapidly heat-inactivated than the former.

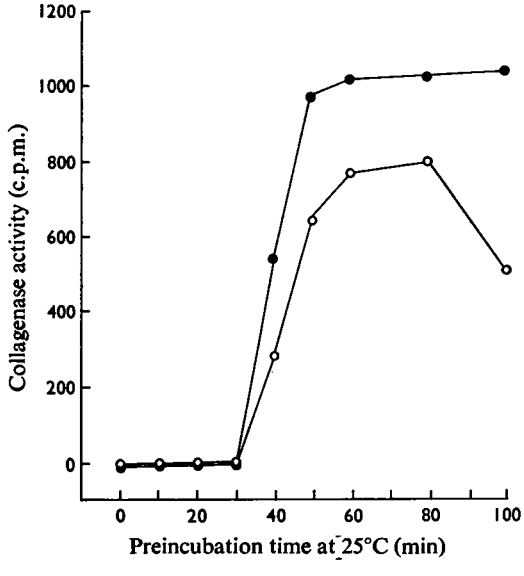

Fig. 5. *Activation of latent collagenase by liver lysosomes*

Purified lysosomes, corresponding to an original 400mg of rat liver tissue, were preincubated at 25°C for the time indicated with 0.2ml of culture medium in a total volume of 0.7ml buffered either at pH5.7 (○) with 142mM-sodium acetate–acetic acid buffer or at pH6.5 (●) with 142mM-sodium cacodylate–HCl buffer; at the end of the preincubation the pH was adjusted to 7.5 by addition of 0.1ml of 0.25M-tris–HCl buffer, pH7.5, containing the appropriate amount of NaOH. Collagenase was then assayed (4h incubation) in these mixtures. No activation of latent collagenase was observed when the pH of the mixtures containing lysosomes, culture medium and buffer was returned to 7.5 before the preincubation at 25°C.

Activation by liver lysosomes

Preincubation at 25°C and at pH5.7 or 6.5 of non-activated medium with purified rat or mouse liver lysosomes resulted in the activation of latent collagenase (Fig. 5); this effect was not observed when the preincubation was done at pH5 or 7.5; no activation was observed by the preincubation of non-activated culture medium alone at any of these pH values. The activation of the enzyme followed a sigmoidal course, as was the case when trypsin was used as activator; the initial lag period was particularly long. More activity was recovered after preincubation at pH6.5 than at 5.7, possibly because a faster rate of collagenase degradation followed the activation when the preincubation was done at pH5.7. The rate of activation was dependent on the concentration of lysosomal extract. The activating factor

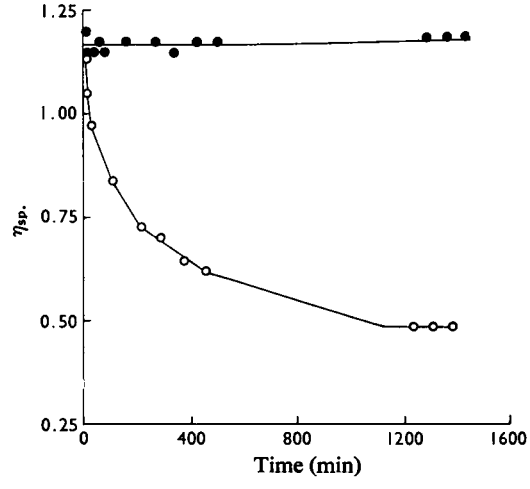

Fig. 6. *Effect of activated culture medium on the viscosity of a collagen solution*

The mixtures were kept at 25°C and the viscosity was measured at the various time-intervals indicated. Activated culture medium was used either as such (○) or, for the control, after having been heated for 15min at 100°C (●). Specific viscosity ($\eta_{sp.}$) remained unchanged in the control during the 24h period of the assay.

present in the lysosomes was labile: it was lost after storage of the lysosomal extract for 8 days at −20°C.

Properties of activated bone collagenase

Activated collagenase attacks trypsin-resistant, salt-soluble collagen molecules either in solution or in fibrillar form (Fig. 1). At 25°C, it decreases to less than half its value the specific viscosity of a collagen solution containing approx. 1 mg of collagen/ml (Fig. 6). It also releases soluble hydroxyproline from an insoluble semi-purified preparation of guinea-pig skin collagen that was resistant to trypsin degradation (Fig. 7); a similar effect was observed on a commercial preparation of insoluble bovine achilles-tendon collagen, but this preparation was also significantly degraded by trypsin, a fact indicative of its partial denaturation. From the results presented in Fig. 7, it can be calculated that the activity of bone collagenase on the insoluble guinea-pig skin substrate amounts to the solubilization of 3.1 μg of collagen (0.42 μg of hydroxyproline)/min per ml of culture fluid; it is thus significantly lower than that exerted on collagen molecules in solution (see below).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the degradation products of collagen after incubation with collagenase at 25°C

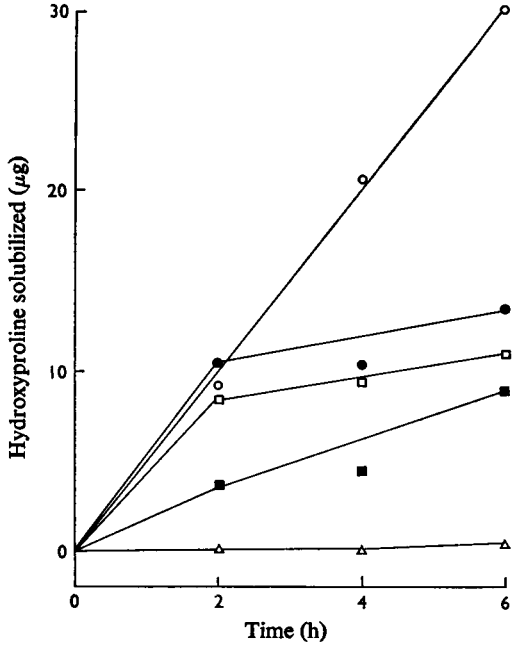

Fig. 7. *Action of bone collagenase and of trypsin on insoluble collagen fibres*

Partially purified insoluble collagen fibres from guinea-pig skin were incubated for the time indicated either with activated (○) or non-activated (Δ) culture medium or with various concentrations of trypsin: 25 μg/ml (■), 100 μg/ml (□) or 500 μg/ml (●). The quantity of hydroxyproline released in soluble form by the action of these agents was then measured. Addition of EDTA to the reaction mixtures at a final concentration of 20 mM completely abolished the action of activated culture medium but did not affect significantly that of trypsin.

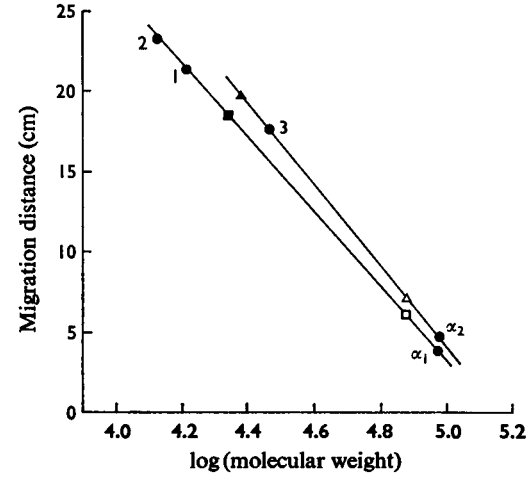

Fig. 8. *Determination of the molecular weights of collagenase degradation products of collagen by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis*

A collagen solution was incubated for 10 h at 25°C with trypsin-activated bone culture medium; the reaction mixture was then dialysed against 0.1 M-acetic acid and freeze-dried. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis for 5 h on a 50 μg sample of the freeze-dried material was done with a 7.5% (w/v) acrylamide gel. The molecular weights of the unknowns have been evaluated by the method of Furthmayr & Timpl (1971) from a plot of electrophoretic mobility (migration distance in cm from the origin) against the log of the molecular weight of the standards; separate calibration curves were used for the α_1- and for the α_2-derivatives. Standards used for calibration (●) include uncleaved α_1 and α_2 chains (mol.wt. 95000) and some of their CNBr peptides (see Miller *et al.*, 1969): α_1 CB6 peptide, of mol.wt. 16500 (1); α_1 CB3 peptide, of mol.wt. 13500 (2); α_2 CB3 peptide, of mol.wt. 29500 (3). Unknowns: α_1 large fragment, □; α_1 small fragment, ■; α_2 large fragment, Δ; α_2 small fragment, ▲.

for increasing times of incubation shows the progressive transformation of the various kinds of monomers, dimers and higher polymers of collagen into smaller molecular pieces with no observable change in the controls incubated with heat-inactivated culture medium. For the monomers, these pieces were mainly represented by a double fraction migrating close to the bottom of the gel and by a second double fraction located ahead of the monomers. Measurements made with reference to the standards give a mol.wt. of 76000 for the large fragments of the two types of α-chains (α_1 and α_2) and a mol.wt. of 22000 to 24000 for the corresponding small fragments (Fig. 8). As the sums of large and small fragments are at least equivalent to the molecular size of the uncleaved chains, it can be assumed that the collagenase cleaves the collagen molecule at only one location on the three α-chains to give fragments representing approx. three-quarters and one-quarter of the molecule.

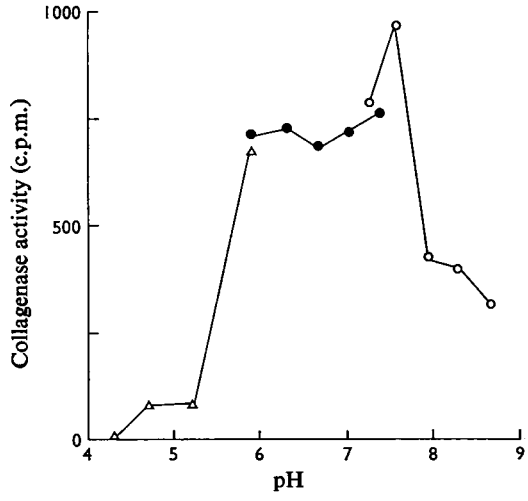

Fig. 9. *Influence of pH on the collagenase activity (gel assays) of activated culture medium*

Incubations were done for 150min in 50mM concentrations of the following buffers: sodium acetate–acetic acid (△), sodium cacodylate–HCl (●), tris–HCl (○).

Collagenase activity is optimum between pH6 and 8 when it is assayed on reconstituted collagen gels (Fig. 9). The activity is lost by ultradialysis of the activated culture medium but is then recovered by the addition of Ca^{2+}; it is inhibited by EDTA, human serum and cysteine but not by soya-bean trypsin inhibitor (Table 1). In the absence of substrate, latent as well as activated collagenase are reasonably stable in the culture medium at 0°C between pH4 and 7–7.5 but at 37°C, activated collagenase is rapidly lost at all pH values, whereas the latent enzyme is stable only between pH5.5 and 7 (Fig. 10).

The enzyme activity obeyed Michaelis–Menten kinetics. K_m values determined on collagen solutions at 25°C were between 0.5 and 0.6mg of collagen/ml; a similar K_m was obtained after activation of the culture

Table 1. *Effect of various agents on bone collagenase activity*

Collagenase assays were done on culture medium either before or after its extensive ultradialysis (in an Amicon Diaflo UM-10 membrane) against 50mM-tris–HCl buffer, pH7.5. In Expts. A and B, EDTA, $CaCl_2$ or soya-bean trypsin inhibitor was added to the assays at the concentration indicated. In Expt. C, the culture medium was preincubated for 90min at 0°C together with 1% or 10% (v/v) of human serum or with 2.5M-cysteine; the assays were done thereafter on these mixtures in the presence of half these concentrations, as indicated.

	Reagent		Collagenase activity (% of control)	
Expt.	Preincubation	Assay	Before dialysis	After dialysis
A	—	—	100	24
	—	EDTA, 0.1mM	75	0
	—	EDTA, 0.25mM	43	0
	—	EDTA, 1mM	0	0
	—	$CaCl_2$, 0.25mM	100	92
	—	$CaCl_2$, 1mM	107	102
	—	$CaCl_2$, 5mM	103	98
B	—	Soya-bean inhibitor, 50μg/ml	107	—
C	Serum, 1%	Serum, 0.5%	92	—
	Serum, 10%	Serum, 5%	17	—
	Cysteine, 2.5mM	Cysteine, 1.25mM	48	—

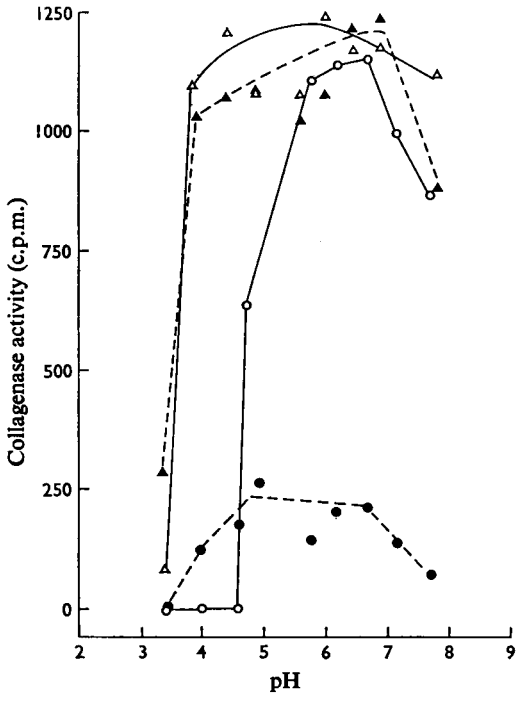

Fig. 10. *Influence of pH on the stability of activated and latent collagenase at 0°C and at 37°C*

Non-activated (open symbols and solid lines) or trypsin-activated culture medium (full symbols and broken lines) were preincubated, in the absence of substrate, either for 3h at 0°C (△,▲) or for 2h at 37°C (○, ●) in the presence of 0.33M concentrations of the following buffers: glycine–HCl (pH3.45 values), sodium acetate–acetic acid (pH4–5.75 values), sodium cacodylate–HCl (pH6.2–7.15 values) or tris–HCl (pH7.7 values). At the end of the preincubation, the pH was adjusted to 7.7 by addition of 0.33 vol. of 0.25M-tris–HCl buffer, pH7.5, containing the appropriate amount of NaOH and supplemented with NaCl to prevent large variations in the ionic strength. Collagenase was then assayed in the mixtures, either directly or after preliminary activation by trypsin.

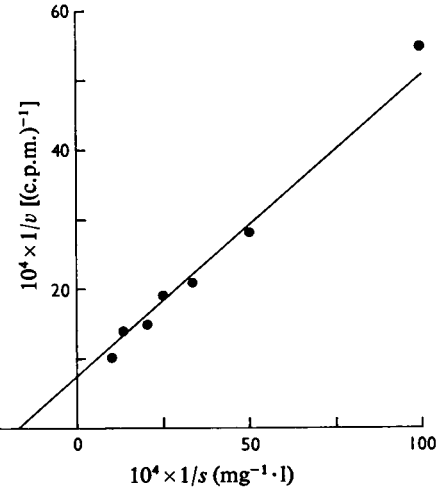

Fig. 11. *Influence of substrate concentration on the velocity of the collagenase reaction: Lineweaver–Burk plot*

medium by chymotrypsin. The $V_{max.}$, measured on medium from the first four days of culture, was 66μg of collagen degraded/min per ml of culture medium (Fig. 11).

Gel filtration of culture media

A single peak of collagenase activity, well separated from the bulk of the proteins, was recovered after gel filtration on Sephadex G-200 of either non-activated (Fig. 12*a*) or activated culture media (Fig. 12*b*). Gel filtration either on Sephadex G-200 (Fig. 12*a*) or on Sephadex G-100 (see below: Fig. 15) did not activate the latent collagenase from non-activated culture media, even when the fractions containing the latent enzyme were pooled together, reconcentrated by ultrafiltration and passed again through a Sephadex G-200 column. As is apparent from Fig. 12, the appearance of the activated collagenase in the effluent from the Sephadex G-200 column was slightly retarded as compared with that of the latent enzyme; this was a reproducible result, which could also be obtained when mixtures of activated and non-activated culture media were passed through the column. By comparison with the results obtained by calibration of the Sephadex G-200 column, the molecular weight of the latent collagenase was 105000±9800 (mean±S.D.) and that of the activated enzyme was 83700±7000 (Fig. 13).

Inhibitors of collagenase in the culture media

Addition of 1–3vol. of non-activated culture media to activated media usually did not inhibit the collagenase present in the activated media when Eagle's basal medium was used as culture fluid. A weak inhibition was, however, often observed when Dulbecco's modified Eagle's medium was used: the inhibitor was as a rule more active in the media from the first 4 days of culture than later; it was thermo-

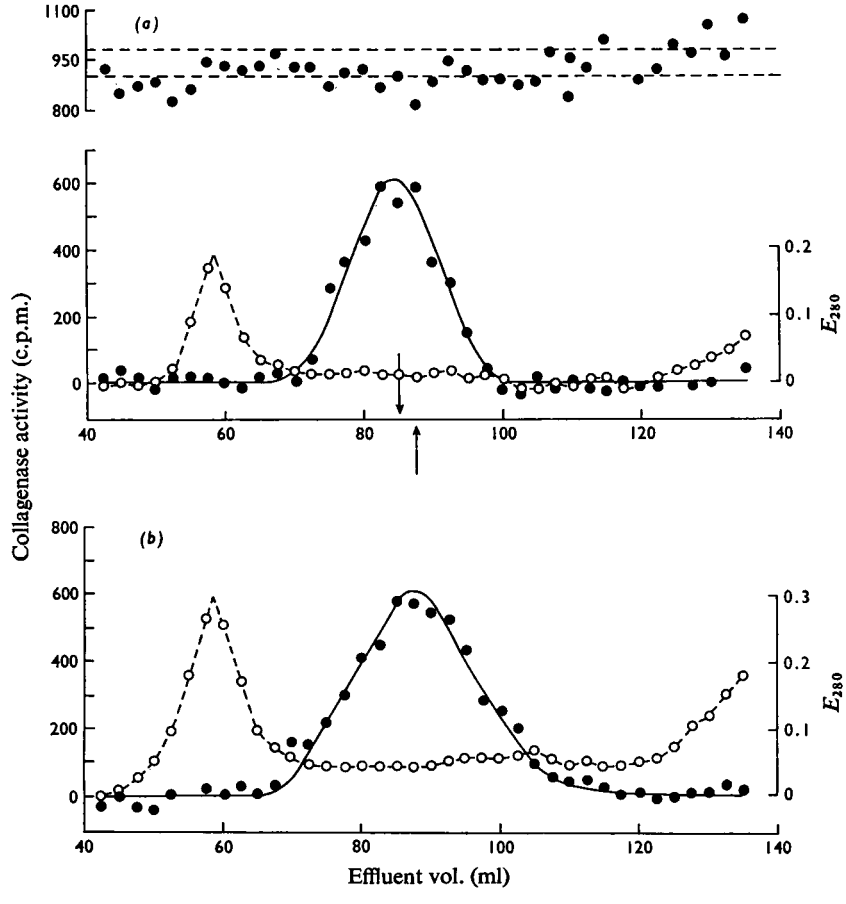

Fig. 12. *Gel filtration of culture media on Sephadex G-200*

●, Collagenase activity; ○, E_{280}. (*a*) Non-activated media. Collagenase assays were done after trypsin activation of the fractions; no directly measurable collagenase activity was found in the fractions when they were not activated by trypsin. The upper graph shows assays for possible collagenase inhibitors (fractions were preincubated for 1 h at 0°C with 0.25 vol. of activated culture medium before the assays); the horizontal lines indicate the limits of variability in normal assays (activated culture medium preincubated with the eluent). (*b*) Trypsin-activated media. The arrows indicate the positions of the peaks of the eluted collagenase activities. The void volume of the column was 57 ml.

stable, trypsin-resistant and non-competitive. Combinations of activated enzyme with that inhibitor were not reactivated when they were incubated with an excess of trypsin (Fig. 14). No clear evidence for the presence of an inhibitor in the effluent from the Sephadex G-200 column was found in the experiment shown in Fig. 12(*a*). Such an inhibitor was, however, found in the effluent from a Sephadex G-100 column in another series of experiments (Fig. 15); it was then located at or very close to the exclusion limit of the gel and its molecular weight was estimated, after calibration of the column, to be $\geqslant$120 000.

Attempts to activate the latent collagenase non-enzymically

As already mentioned, gel filtration of the culture media did not activate the collagenase. Also, the collagenase remained latent in a fraction precipitated from the media at 4°C by $(NH_4)_2SO_4$ between 20 and 50% saturation and dialysed extensively thereafter against 50 mM-tris–HCl buffer, pH 7.5, containing 5 mM-$CaCl_2$. Up to 100-fold dilution of the non-activated culture medium did not cause any activation, nor did a 1600-fold dilution obtained by ultradialysis of the medium at pH 7.7 on Amicon Diaflo

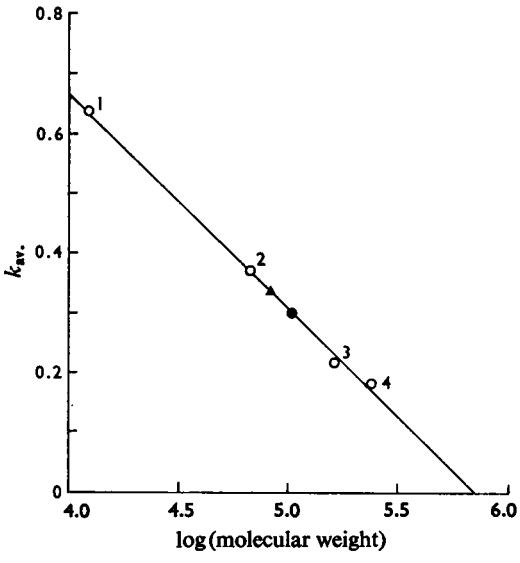

Fig. 13. *Determination of the approximate molecular weights of latent and of activated bone collagenase from their elution behaviour on a calibrated Sephadex G-200 column*

●, Latent bone collagenase; ▲, activated bone collagenase. As described by Fischer (1971), the molecular weight of the unknowns has been evaluated from a plot of $K_{av.}$ [$=(V_e-V_0)/(V_t-V_0)$] against the log of the molecular weight of the proteins used for calibration (1, cytochrome *c*; 2, serum albumin; 3, γ-globulin; 4, catalase). V_e, elution volume; V_0, void volume (57ml); V_t, total bed volume (155ml). The values presented for the latent and for the activated collagenase are the means of three determinations each; the difference between the molecular weight of latent and of activated collagenase is significant at $P<0.05$ with Student's *t* test.

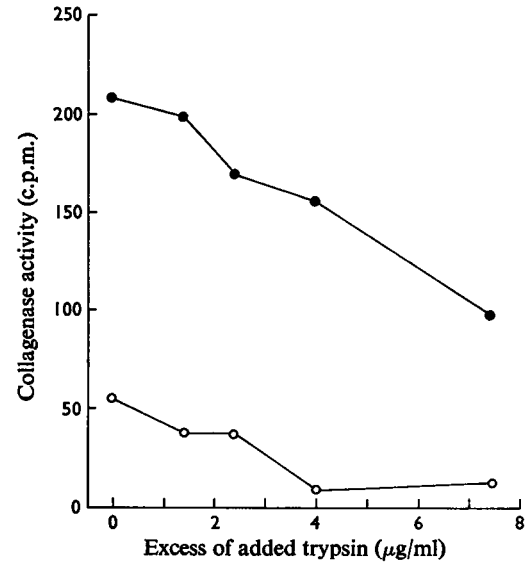

Fig. 14. *Attempt to reactivate with trypsin a combination of activated collagenase with its inhibitor*

Fully activated culture fluid (1vol.) was mixed with 3vol. either of a culture medium (Dulbecco's) that contained an inhibitor of the collagenase (○) or of 50mM-tris–HCl buffer, pH7.5, containing 1mM-$CaCl_2$ (●); the mixtures were preincubated for 45min at 0°C. Then excess of trypsin was added to overcome the action of the soya-bean trypsin inhibitor contained in the fully activated culture fluid; it was calculated on the basis that 1mg of the soya-bean inhibitor will neutralize the action of 1.6mg of trypsin. The mixtures were further incubated at 25°C for 10min, after which an excess of soya-bean inhibitor was added to block the action of trypsin. Collagenase was then assayed in the various mixtures. As seen on the graph, excess of trypsin inactivates progressively the collagenase but does not reactivate the enzyme that was inhibited by the inhibitor present in Dulbecco's medium.

XM-50 membrane. Moreover, no activation was obtained by ultradialysis at 4°C on XM-50 or on PM-30 membranes either at various pH values between pH2.5 and 10, or at pH7.7 in the presence of various additives: 5 or 10M-urea, 0.1 or 1mg of Triton X-100/ml or 3.5M-NaCl. All these treatments left at least part of the activity of trypsin-activated collagenase.

Discussion

The collagenolytic enzyme found in a latent state in the culture fluid surrounding bone explants in the present experiments is a true collagenase, as defined by Gross (1970): it attacks native, trypsin-resistant collagen molecules at neutral pH values in solution or in reconstituted fibrils and liberates soluble hydroxyproline from insoluble collagen fibres; it decreases the intrinsic viscosity of collagen solutions by more than 50% and produces degradation products of collagen (two large fragments corresponding to approx. 75 and 25% of the molecule) reminiscent of those obtained with tadpole collagenase (Sakai & Gross, 1967); moreover, it requires Ca^{2+} for its activity and is inhibited by EDTA, cysteine and human serum, as are most of the collagenases isolated to date. The enzyme is thus most probably identical with the collagenase isolated from the

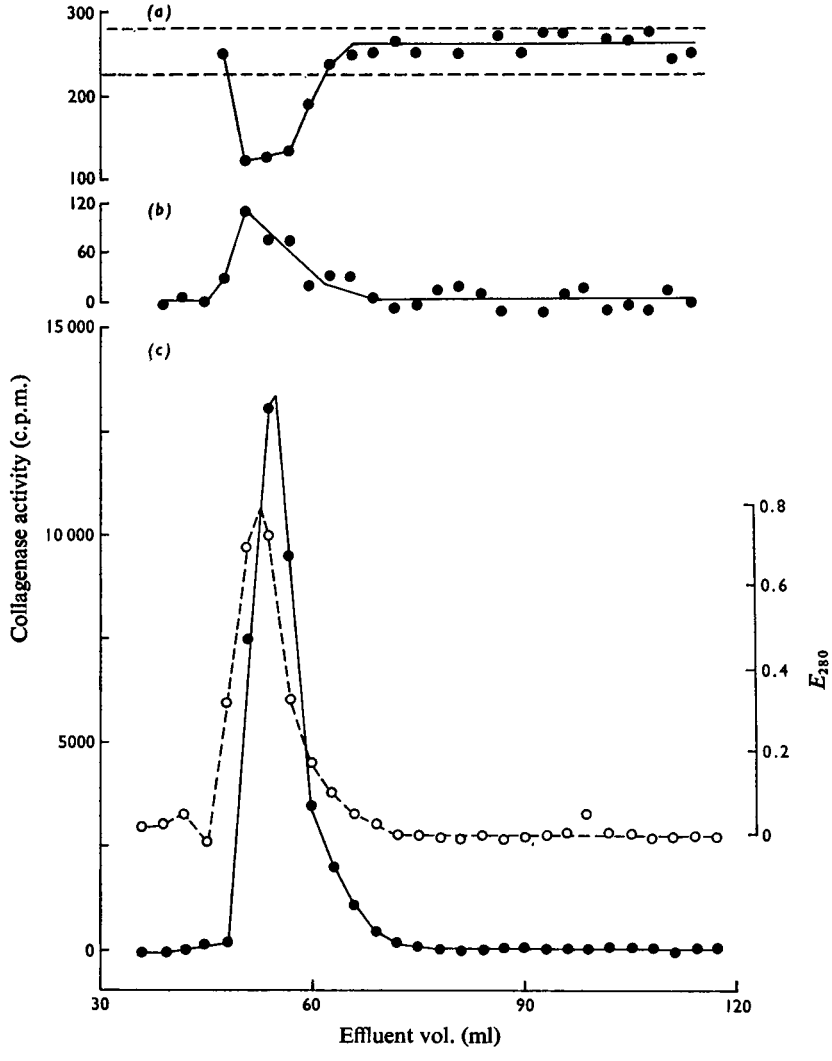

Fig. 15. *Gel filtration of culture medium on Sephadex G-100*

Non-activated, concentrated culture medium was chromatographed as described in the Experimental section. Several assays were done on the effluent fractions: detection of collagenase inhibitors (*a*), as described in Fig. 12 (the limits of the normal collagenase activity are given by the horizontal broken lines); direct assay of active collagenase (*b*); assay of collagenase after activation of the fractions with trypsin (*c*). Note the difference in scale between the ordinates of (*b*) and (*c*). The void volume of the column was 52ml. Collagenase activity, ●; E_{280}, ○.

medium surrounding human, rat or mouse bones in culture (Fullmer & Lazarus, 1967, 1969; Shimizu *et al.*, 1969), but in these studies, collagenase activity was demonstrated only in concentrated, pooled culture media. In the present experiments, non-concentrated culture media, which were almost inactive when assayed directly, displayed considerable collagenase activity when they had first been exposed to trypsin or to other proteolytic enzymes. Also, the molecular weight of activated mouse bone collagenase was approximately 84000 in our experiments but 41000 in experiments by S. Sakamoto, P. Goldhaber & M. J. Glimcher (S. Sakamoto, personal communication): this 2-to-1 relationship may indicate that collagenase may exist in polymeric form, as already suggested by the demonstration of

two distinct collagenases, with respective mol.wt. of 20000–25000 and of 40000–50000, in human synovial fluid (Harris *et al.*, 1969).

The latency of the collagenase found in the culture media could be explained by two mechanisms: (1) either collagenase is released from the cells as an inactive proenzyme, a 'pro'-collagenase or a 'pre'-collagenase, that is activated after a limited proteolysis of its molecule, or (2) collagenase forms an enzyme–inhibitor complex with some protein inhibitor, which is dissociated after partial proteolysis by trypsin. If the proenzyme hypothesis is correct activation would presumably be due to the rupture of covalent bonds in one single molecule; if the enzyme–inhibitor-complex hypothesis is correct activation would be due to the dissociation of two proteins linked together by non-covalent bonds. From the gel-filtration experiments, it appears that the molecular weight of the latent collagenase is changed from 105000 to 84000 by the activation process, indicating the loss of a fragment of mol.wt. 21000.

It latency were due to an enzyme–inhibitor complex, the inhibitor would have to be in a tight stoicheiometric relation with the enzyme, as we were unable to detect an excess of inhibitor in most of the culture media. This stoicheiometry could suggest that the inhibitor is of physiological significance and that the enzyme–inhibitor complex is formed inside the cell, before the secretion of the latent enzyme. The distinction between a classical proenzyme and such an enzyme–inhibitor complex would then become very tenuous, depending only on the covalent or non-covalent nature of the bonds between the enzyme and the associated peptide.

A weak non-competitive inhibitor of collagenase could, however, be found in some culture fluids, but it was resistant to trypsin, and complexes formed by the activated collagenase and that inhibitor were not reactivated by trypsin. This indicates that the latency of collagenase in the culture media, which is overcome by trypsin, is unlikely to be due to such a complex. Moreover, the only inhibitor that could be localized in the effluent fractions after gel filtration of non-activated culture media had a molecular weight $\geqslant$120000 so that, again, the latency of the collagenase cannot be explained by the association of active enzyme with that inhibitor, as the molecular weight of the latent enzyme cannot account for such an association.

Eisen *et al.* (1971) have found an active collagenase in the effluent fractions obtained after gel filtration on Sephadex G-150 of a human skin extract that, before fractionation, was devoid of any collagenase activity. The latency of the collagenase in the whole skin extracts was due to the presence of two protease inhibitors, α_1-anti-trypsin and α_2-macroglobulin, which were separated from the enzyme by the chromatography. Our results are unlikely to be explicable by such a mechanism: gel filtration of the culture media on Sephadex G-200 did not cause any activation of the collagenase, even when the fractions containing the latent enzyme were reconcentrated and chromatographed again on the column. Moreover, the molecular weight of α_1-anti-trypsin (45000) and of α_2-macroglobulin (>800000) are not compatible with the difference in molecular weight (21000) observed between latent and active collagenase in the present experiments.

Finally, the peptide of mol.wt. 21000 that is separated from the latent collagenase by trypsin should be able to pass through the Amicon Diaflo ultrafilters PM-30 (retention limit about mol.wt. 30000) or XM-50 (retention limit about mol.wt. 50000), but active collagenase (mol.wt. 84000) should be mostly retained by such filters. However, extensive ultradialysis in these membranes, resulting in a 1600-fold dilution of the culture medium, did not activate the latent collagenase, as would be expected if the latency were due to an equilibrium between the enzyme, the inhibitor and an enzyme–inhibitor complex. Also, changing the pH from 10 to 2.5, or increasing the ionic strength by addition of 3.5M-NaCl, or addition of up to 10M-urea or of up to 1mg of Triton X-100 detergent/ml did not cause the activation of the latent collagenase when culture media were ultradialysed on these membranes, indicating again that the latency is unlikely to be due to the non-covalent association of collagenase with an inhibitor peptide.

From all this evidence, it is reasonable to conclude that the release of collagenase as an inactive precursor or proenzyme (a 'procollagenase') by the cells is the most probable explanation for the latency observed for that enzyme in the present experiments; but the final proof of this hypothesis can only result from studies made on completely purified latent collagenase. The physiological importance of such a mechanism is evident as it should allow, together with the inhibition of the enzyme by serum antiproteases elegantly demonstrated by Eisen *et al.* (1971), a delicate regulation of the extracellular activity of collagenase (a very dangerous enzyme, indeed, for the organism!) by interactions involving not only the production of procollagenase but also its activation. Moreover, the presence of collagenase as an inactive proenzyme in cells could also explain why it has not been possible so far to demonstrate collagenase in most cell extracts. It is noteworthy that Goldstein *et al.* (1964) have reported that trypsin treatment at pH5.5 caused the appearance of a collagenolytic factor in iso-osmotic saline extracts of rat skin. This factor is, however, clearly different from the collagenase studied in the present experiments, as it was not inhibited by EDTA and had a pH optimum at 5.5 with no activity at pH7.5; it could be related to the collagenolytic cathepsin found in rat subcutaneous granulomas by Bazin & Delaunay (1971). The mechanism of its latency was not investigated.

The search for proteases acting physiologically as activators of procollagenase becomes of prime importance, as the activity of these proteases will condition that of the collagenase. In this regard, the fact that purified lysosomes are able to activate collagenase at neutral pH values is of interest. Lysosomal enzymes appear to participate in the extracellular degradation of several connective tissues (Woessner, 1968), including bone (Vaes, 1968, 1969), where the action of a true collagenase has also been implicated and it is likely that part of their action is to activate procollagenase.

Whereas trypsin-activated culture medium is unable to activate latent collagenase, culture fluids that have been 'sub-activated' by a very limited treatment with trypsin that is insufficient to elicit their latent collagenase activity became progressively active when they were further incubated at 25°C, and this activation could be attributed to a thermolabile agent that is not collagenase. This indicates strongly that collagenase is not able to activate itself by autocatalysis but that there exists in the culture media an agent that is able to activate the latent collagenase and that is also elicited by trypsin activation. Preliminary results of experiments to study the nature of this agent further (G. Vaes, unpublished work) are compatible with its identification as a distinct protease active at neutral pH, which was found in a latent state in the media and which is also activated by trypsin, but at a faster rate than is the collagenase. The mechanism of the latency and of the activation of this protease as well as its nature remain to be elucidated; also the possibility should be considered that the lower efficiency of chymotrypsin to activate the latent collagenase, when compared with trypsin, is because chymotrypsin acts only indirectly on the collagenase, through the activation of that particular activator protease.

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