Further Studies on the Activation of Procollagenase, the Latent Precursor of Bone Collagenase

EFFECTS OF LYSOSOMAL CATHEPSIN B, PLASMIN AND KALLIKREIN, AND SPONTANEOUS ACTIVATION

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1. Cathepsin B, a tissue (lysosomal) proteinase, and two humoral proteinases, plasmin and kallikrein, activate the latent collagenase ('procollagenase') which is released by mouse bone explants in culture. Other lysosomal proteinases (carboxypeptidase B, cathepsin C and D) and thrombin did not activate the procollagenase. Dialysis of the culture fluids against 3M-NaSCN at 4°C and, for some culture fluids, prolonged preincubation at 25°C also caused the activation of procollagenase. 2. In all these cases, activation of procollagenase involved at least two successive steps: the activation of an endogenous latent activator present in the culture fluids and the activation of procollagenase itself. 3. An assay method was developed for the endogenous activator. Human serum, bovine serum albumin, casein and cysteine inhibited the endogenous activator at concentrations that did not influence the collagenase activity. *N*-Ethylmaleimide and 4-hydroxy-mercuribenzoate stimulated the endogenous activator, but iodoacetate had no effect. 4. It is proposed that cathepsin B, kallikrein and plasmin may play a role in the physiological activation of latent collagenase and thus initiate degradation of collagen *in vivo*. This may occur whatever the molecular nature of procollagenase (zymogen or enzyme-inhibitor complex) might be.

Neutral collagenases (EC 3.4.24.3) are implicated in several physiological or pathological processes (e.g. bone resorption) involving connective-tissue breakdown (for a review, see Harris & Krane, 1974). Previous work (Vaes, 1971, 1972a) has established that mouse bone and skin explants release into the surrounding culture fluids a latent collagenase, which can be activated by limited proteolysis, e.g. by trypsin. Evidence was presented supporting the view that this latent collagenase represents an inactive precursor of the enzyme, a 'procollagenase'. Moreover it was observed (Vaes, 1972b) that the action of trypsin is exerted indirectly through the activation of a latent enzyme (or 'endogenous activator') which is able to activate the procollagenase and which is also present in the culture fluids.

These observations indicate that the extracellular activity of collagenase might be controlled by another proteinase (or even possibly by an enzyme cascade) that activates the procollagenase. As it is unlikely that trypsin acts as a physiological activator of procollagenase *in vivo*, a search was made for other tissue or humoral proteinases that could act as activators. The present report establishes that the lysosomal enzyme cathepsin B (EC 3.4.22.1), and also

plasmin (EC 3.4.21.7) and kallikrein (EC 3.4.21.8), can activate the procollagenase and that they also act indirectly through the activation of the latent endogenous activator that is present in the culture fluids. An assay method for that endogenous activator is also presented.

Part of the work has been presented as preliminary notes (Eeckhout & Vaes, 1974; Eeckhout *et al.*, 1974) or in the context of a general review on procollagenase (Vaes & Eeckhout, 1975a).

Experimental

Methods

Whole tibiae from 5-day-old NMRI mice were cultivated in serum-free Eagle's basal medium as described by Vaes (1972a). All experiments were done on culture fluids harvested after 4 days. Collection and handling of the media (including sometimes their concentration by ultrafiltration on Amicon Diaflo UM-10 or PM-30 membranes) and their activation by trypsin were performed as described earlier (Vaes, 1972a). 'Subactivated' media (i.e. culture media that have been in limited contact with exogenous activator, e.g. trypsin, insufficient to elicit

any significant collagenase activity from its precursor) were prepared as described by Vaes (1972*b*) for trypsin or as described in the Results section for the other exogenous activators. Except when otherwise stated, maximum collagenase activity elicited by the various activators did not differ significantly from the maximum collagenase activity obtained in the same culture fluid after complete trypsin activation. However, maximum collagenase activity and length of lag phase differed from one culture fluid to the other.

Collagenase assays were done on radioactive soluble collagen as described by Vaes (1972*a*). Their results are expressed either as the radioactivity of the supernatant obtained after sedimentation of the residual collagen fibrils or in units/ml of culture fluid, one unit referring to the degradation of $1\mu\text{g}$ of native soluble collagen/min at 25°C (this corresponds approximately to the degradation of $1\mu\text{g}$ of reconstituted collagen fibrils/min at 37°C). One unit of procollagenase refers to the amount of latent enzyme that gives rise to 1 unit of collagenase activity after its full activation by trypsin. When collagenase activity is expressed in c.p.m., it corresponds to the radioactivity liberated by $50\mu\text{l}$ of enzyme preparation incubated for the indicated time with $50\mu\text{g}$ of collagen (1000–2500 c.p.m. according to the preparation) in a total volume of $100\mu\text{l}$.

Purified liver lysosomes were prepared by the method of Trouet (1974) from rats that had been injected with Triton WR-1339. These preparations contained 0.35% of the total proteins of the tissue (i.e. 0.7 mg of protein/g original wet wt. of liver) as well as 14% of the activity of its total lysosomal hydrolases; a 40-fold purification was thus achieved. The purified lysosomes were always frozen and thawed three times (in a mixture of solid CO_2 and propan-2-ol) to rupture their membrane and to allow the action of the lysosomal enzymes on their substrates.

Just before their use, the solutions of cathepsin C, cathepsin B and of carboxypeptidase B were desalted on a Sephadex G-25 column equilibrated with a 10 mM-sodium cacodylate/HCl buffer, pH 6.5, containing 0.15 M-NaCl and 0.5 mM-dithiothreitol.

Di-isopropyl phosphorofluoridate (Dip-F) and phenylmethanesulphonyl fluoride solutions were prepared in propan-2-ol.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Materials

L-Cysteine, 2-mercaptoethanol, propan-2-ol, phenylmethanesulphonyl fluoride and sodium thiocyanate were obtained from Merck A.G. (Darmstadt, Germany). Dithiothreitol, 4-hydroxymercuribenzoate (sodium salt), iodoacetic acid, *N*-ethylmaleimide,

Tris, α -*N*-benzoyl-L-arginine amide, α -*N*-benzoyl-DL-arginyl-2-naphthylamide, Tos-Lys- CH_2Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 'TLCK'), ox pancreatic trypsin inhibitor, bacterial fibrinolysin (EC 3.4.21.7; 1.2 units/mg), bovine thrombin (EC 3.4.21.5; 400 NIH units/mg), papain (EC 3.4.22.2; twice crystallized; 30 Bz-Arg-OEt units/mg) and cathepsin C (EC 3.4.14.1; 42 units/mg) were from Sigma Chemical Co., St Louis, MO, U.S.A. Trypsin (EC 3.4.21.4; twice or thrice crystallized) and soyabean trypsin inhibitor were from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and casein (Hammarsten quality) was from Nutritional Biochemicals, Cleveland, OH, U.S.A. Pig pancreatic kallikrein (1200 K units/mg) and aprotinin (Trasylol, 5400 K units/mg) were given by Bayer A.G., Wuppertal, Germany. Di-isopropyl phosphorofluoridate and bovine blood plasmin were from Koch-Light, Colnbrook, Bucks., U.K., and human blood plasmin (15 casein units/mg) was from AB Kabi, Stockholm, Sweden.

Purified human liver cathepsin D (EC 3.4.23.5; 277 units/mg) was a gift of Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge, U.K. (Barrett, 1970). Cathepsin B (formerly cathepsin B1) and carboxypeptidase B (EC 3.4.12.3; formerly cathepsin B2) were purified respectively 250-fold (0.2 unit/mg) and 1100-fold (5 units/mg) from ox spleen (Otto & Riesenköning, 1975) and given by Dr. K. Otto, University of Bonn, Germany.

All other chemicals and biochemicals were commercially available analytical-grade reagents.

Units of the commercial enzyme preparations are those used by their respective suppliers. One unit of bacterial fibrinolysin will release $1\mu\text{mol}$ of tyrosine/min at pH 7.4 at 37°C , with α -casein as substrate. One NIH unit of thrombin will clot a standard fibrinogen solution in 15 s at 37°C . One unit of papain will hydrolyse $1\mu\text{mol}$ of α -*N*-benzoyl-L-arginine ethyl ester/min at pH 6.2 at 25°C . One unit of cathepsin C will produce $1\mu\text{mol}$ of hydroxamic acid from glycyl-L-phenylalanine amide and hydroxylamine/min at pH 6.8 at 37°C using DL-phenylalanine hydroxamate as the standard. One kallikrein unit (K unit) corresponds to the kallikrein activity present in 5 ml of dialysed normal human urine. One kallikrein inhibitor unit (KI unit) is the amount that inhibits 2 K units by 50%. The casein unit of plasmin is defined by Robbins & Summari (1970). Cathepsin B and carboxypeptidase B units are defined by Otto & Riesenköning (1975); one unit of cathepsin D corresponds to the definition given by Barrett (1970).

Results

Properties and nature of a lysosomal activator of procollagenase

As already observed (Vaes, 1972*a*), purified liver

lysosomes were able to activate the latent collagenase released by bone explants in culture. When culture medium was incubated with increasing concentrations of purified lysosomes (see Fig. 2 in Vaes & Eeckhout, 1975a), the activation of procollagenase followed a sigmoidal course; the latency period preceding this activation was inversely related to the concentration of lysosomes, but the rate of the activation proper was independent of that concentration. This activating capacity was lost during preincubation of the lysosomes for 10 min at 100°C or during storage at -20°C.

Fresh lysosomal preparations (Fig. 1a) were more active in the presence of dithiothreitol and were inhibited by the thiol-blocking agents 4-hydroxymercuribenzoate (0.1 mM), iodoacetate (10 mM) and *N*-ethylmaleimide (5 mM), as well as casein (5 mg/ml) or by α -*N*-benzoyl-L-arginine amide (20 mM), a substrate of cathepsin B and of carboxypeptidase B. When the activating capacity of purified lysosomes had been lost during storage at -20°C, it could be restored by the addition of thiol compounds such as cysteine, mercaptoethanol or dithiothreitol (Fig. 1b); in that case, the activation of procollagenase also followed a sigmoidal course and was preceded by a period of latency that was then inversely related to the concentration of dithiothreitol. It was verified that under these conditions none of the compounds tested exerted any significant influence on the collagenase activity of trypsin-activated culture medium and also that the lysosomal preparation did not display any collagenolytic activity.

The activation of latent collagenase by purified lysosomes was pH-dependent (Fig. 2). Optimum yields of collagenase were obtained when procollagenase had been incubated with the lysosomal extract at pH 6.5; this resulted both from a complete activation and from the lack of degradation of collagenase by the lysosomal extract at that pH. At higher pH the activation was slowed down, whereas at lower pH collagenase and procollagenase were rapidly degraded by the lysosomes, so that it was not possible to determine precisely the optimum pH of the lysosomal activator.

To help determine the nature of the proteinase that was responsible for the activating capacity of purified lysosomes, the influence of two purified thiol-sensitive cathepsins (B and C) was examined as well as that of carboxypeptidase B and of cathepsin D. Only cathepsin B activated latent collagenase (Fig. 3a) and stimulated its activation by purified lysosomes (Fig. 3b). This enzyme did not display any collagenase activity under the conditions of our collagenase assay; it did, however, progressively inactivate collagenase (Fig. 3a). Cathepsin C had no effects (Figs. 3a and 3b), nor did carboxypeptidase B (0.34 unit/ml) and cathepsin D (36 units/ml); this last enzyme did, however, rapidly inactivate collagenase (Fig. 3b). Papain (0.2 unit/ml), another thiol proteinase, was also able to activate latent collagenase.

Activation of procollagenase by kallikrein and plasmin

Preincubation of culture medium with either hog pancreatic kallikrein or human plasmin resulted in the

Fig. 1. Influence of dithiothreitol, thiol-blocking agents and casein on the activation of procollagenase by purified lysosomes (a) Freshly purified lysosomes, corresponding to an original 400 mg of rat liver tissue, were frozen and thawed and diluted to a final volume of 0.25 ml with 0.2 M-sodium cacodylate/HCl buffer at pH 6.5 (control, ●) supplemented with 14 mM-iodoacetate (Δ), 0.14 mM-4-hydroxymercuribenzoate (▲), 7 mg of casein/ml (■), 7 mM-*N*-ethylmaleimide (□) or 1.4 mM-dithiothreitol (○). (b) Purified lysosomes stored for 1 week at -20°C, corresponding to an original 200 mg of liver, were diluted to a final volume of 0.25 ml with 0.2 M-sodium cacodylate/HCl buffer, pH 6.5, either in the absence (control, ●) or in the presence of various concentrations of dithiothreitol: 0.35 mM (Δ), 0.70 mM (▲) or 1.4 mM (○). In both (a) and (b) experiments, 0.1 ml of culture medium was added to each lysosomal suspension and preincubated at 25°C for the time indicated. At the end of that time the pH was adjusted to 7.7 by addition of 0.05 ml of 0.25 M-Tris/HCl buffer containing the appropriate amount of NaOH. Collagenase was then assayed (210 min incubation) in these mixtures.

Fig. 2. Effect of pH on the activation of procollagenase by purified lysosomes

Purified lysosomes corresponding to an original 400 mg of rat liver tissue were preincubated at 25°C for the time indicated with 0.1 ml of culture medium in a total volume of 0.35 ml containing 1 mM-dithiothreitol and either 142 mM-sodium cacodylate/HCl buffer at pH 5.5 (●), 6.0 (■), 6.5 (□) and 7.0 (○), or 142 mM-Tris/HCl buffer at pH 7.7 (▲). At the end of the preincubation the pH was adjusted to 7.7 by the addition of 0.05 ml of 0.25 M-Tris/HCl buffer containing the appropriate amount of NaOH. Collagenase was then assayed (210 min of incubation) in these mixtures.

activation of latent collagenase (Fig. 4) in a manner similar to that observed with either trypsin (Vaes, 1972b) or purified lysosomes (a latency period inversely related to the concentration of the activator followed by a sigmoidal activation that is independent of it). The activation by kallikrein was not influenced by soya-bean trypsin inhibitor (2 mg/mg of kallikrein), but it was blocked by Dip-F (10 µmol/mg of kallikrein), ox pancreatic trypsin inhibitor (1 mg/mg of kallikrein) and Trasylol (25000 K units/mg of kallikrein). Bacterial fibrinolysin (29 µg/ml) and bovine blood plasmin (14 µg/ml) also activated procollagenase, but bovine thrombin (18 NIH units/ml) did not.

Activation of procollagenase by non-enzymic treatments and 'autoactivation'

Although several treatments (extensive dilution, chromatography on Sephadex G-200 or G-150, ultradialysis on Amicon Diaflo XM-50 membranes

at pH values ranging from 2.5 to 10 or in the presence of 10 M-urea, 0.1% Triton X-100 or 3.5 M-NaCl) had been shown by Vaes (1972a) not to activate procollagenase, dialysis of culture fluid at 0°C against 3 M-sodium thiocyanate (Table 1) did evoke that activation. Moreover it appeared that some untreated culture fluids activated themselves spontaneously if they were preincubated for long periods (24–72 h) at 25°C. Under these conditions there was no lag phase and collagenase activity steadily increased with time (Fig. 5). For unknown reasons, the procollagenase of most culture fluids did not become activated spontaneously, and when it did, the rate of this 'autoactivation' varied extensively from one culture medium to the other.

Involvement of a latent activator present in the culture medium

As with the activation obtained by trypsin (Vaes, 1972b), the activation of procollagenase by the present enzymic (purified lysosomes, kallikrein or plasmin) or non-enzymic (NaSCN or preincubation at 25°C) treatments appeared to involve at least two steps: the activation of an endogenous latent activator and the activation of procollagenase itself. Indeed a first series of data has shown that the activation of procollagenase by purified lysosomes, kallikrein or plasmin followed a sigmoidal course; the initial lag phase was inversely related to the concentration of exogenous activator, but this concentration did not influence significantly the slopes of the activation curves (see, e.g., Fig. 4). These observations thus suggested that the activation of procollagenase occurred through an indirect mechanism.

More direct evidence supporting the existence of an endogenous activator resulted from a second series of experiments. 'Subactivated' medium (see under 'Methods') activated itself spontaneously, after inhibition of the exogenous activator, during further preincubation at 25°C. As shown in Fig. 6, a short preincubation of culture fluid with purified lysosomes at pH 6.5, insufficient in itself to elicit any collagenase activity from its precursor, followed by a further incubation at pH 7.7 (a pH at which lysosomes do not activate procollagenase: see Fig. 2), evoked the autoactivation of procollagenase after a latency period that was inversely related to the duration of the preincubation at pH 6.5. However, inactivation of collagenase occurred during the incubation at pH 7.7, owing to dithiothreitol; this did not occur at pH 6.5 or when dithiothreitol was added immediately before the collagenase assay. The procollagenase of a culture medium subactivated by kallikrein (Fig. 7a, curve 1) or by plasmin (Fig. 7b, curve 1) was also activated spontaneously during further incubation at 25°C. Similar observations were made with culture fluid subactivated by sodium thiocyanate but,

Fig. 3. Effect of cathepsin B, C and D on latent and on active collagenase

(a) Preincubation of 0.5 unit of purified cathepsin B (● and ○) or cathepsin C (■ and □) or 1.8 units of cathepsin D (▲ and △) was carried out at 25°C for the time indicated in the presence of 0.15 ml of either trypsin-activated (open symbols and dashed curves) or untreated (closed symbols and continuous curves) culture medium in a total volume of 0.35 ml containing 142 mM-sodium cacodylate/HCl buffer, pH 6.5, and 1 mM-dithiothreitol. (b) In two other experiments with untreated culture medium, the mixtures contained purified rat liver lysosomes (corresponding to an original 190 mg of liver tissue) and, in the first experiment, active (●) or heat-inactivated (○) cathepsin B, and in the second experiment, active (■) or heat-inactivated (□) cathepsin C. At the end of the indicated preincubation time the pH was adjusted in both experiments to 7.7, as described in Fig. 1 legend, and collagenase was then assayed (140 min incubation) in these mixtures.

Fig. 4. Activation of procollagenase by kallikrein and plasmin

Culture medium (0.25 ml) was preincubated at 25°C for the indicated time in a final volume of 0.35 ml in the presence of pig pancreatic kallikrein (continuous lines), 29 µg/ml (△), 57 µg/ml (□) or 300 µg/ml (■), or human plasmin (dashed lines), 290 µg/ml (●) or 29 µg/ml (○). The preincubation mixture was buffered at pH 7.7 with 40 mM-Tris/HCl buffer. The activation was stopped by the addition of 10 µl of 0.1 M-Dip-F and collagenase was then assayed (60 min incubation). It was verified that, under the same conditions, kallikrein and plasmin that had been first inhibited by Dip-F did not activate procollagenase.

Table 1. Activation of procollagenase by sodium thiocyanate

Non-activated, fourfold-concentrated culture medium was dialysed for 16 h at 4°C against 1000 vol. of either 50 mM-Tris/HCl buffer, pH 8, containing 0.2 M-NaCl and 5 mM-CaCl₂ (control) or 3 M-NaSCN in the same buffer. Control and NaSCN-treated dialysis sacs were then dialysed for 24 h (against three changes of 1000 vol. of buffer) and assayed for collagenase activity either before (1) or after (2) activation by trypsin. Trypsin-activated culture medium (3) was treated in the same way to check the recovery of collagenase after these treatments. Collagenase activity is expressed in units/ml of culture medium. It was also verified that replacement of NaSCN by 3 M-NaCl did not activate procollagenase.

Culture medium	Collagenase activity		
	Before dialysis	After dialysis	
		Control	NaSCN
(1) Non-activated	0	0.6	19.3
(2) Activated by trypsin after dialysis	—	23.0	21.8
(3) Activated by trypsin before dialysis	23.7	25.3	20.5

as illustrated in Fig. 8, autoactivation was then slower and more or less linear with time.

A third group of results (Table 2) shows that culture fluids that have been activated by lysosomes, kallikrein, plasmin or sodium thiocyanate decreased

Fig. 5. *Spontaneous activation of procollagenase*
Two different culture fluids were supplemented with 0.2mg of NaN_3/ml and preincubated at 25°C for the indicated times. Collagenase was then assayed (60 min incubation) either in the fluids as such (●, Expt. 1; ▲, Expt. 2) or after their activation by trypsin (○, Expt. 1; △, Expt. 2).

Fig. 6. *'Autoactivation' of procollagenase in culture fluids subactivated by purified lysosomes*

Purified lysosomes, corresponding to an original 400mg of rat liver tissue, were first preincubated at 25°C for either 30 (△) or 60 (○) min, with 0.1 ml of fivefold concentrated culture medium, in a total volume of 0.35 ml with 142 mM-sodium cacodylate/HCl buffer, pH 6.5, containing 1 mM-dithiothreitol. At the end of this first preincubation, the pH was adjusted to 7.7 by addition of 0.05 ml of 0.25 M-Tris/HCl buffer containing the appropriate amount of NaOH. These mixtures were then further preincubated at 25°C for the indicated times before the collagenase assays (30 min of incubation). The indicated preincubation times correspond to the sum of the first and of the second preincubation. Controls were maintained at pH 6.5 (●) or at pH 7.7 (□).

Fig. 7. *Influence of trypsin-activated culture fluid on the autoactivation of procollagenase in culture fluids subactivated by kallikrein (a) or plasmin (b)*

Culture medium was either subactivated by its preincubation at 25°C with (a) kallikrein (90 µg/ml during 2 h) or (b) plasmin (90 µg/ml during 20 min) or completely activated by trypsin (4 µg/ml during 10 min). The action of kallikrein, plasmin and trypsin was stopped by the addition of 2 mM-Dip-F. Then 1 vol. of the subactivated culture fluids was preincubated at 25°C for the indicated times either alone (●) or in the presence of 1/27 (○) or 1/5 (△) vol. of trypsin-activated culture fluid. Collagenase was then assayed (30 min of incubation) in these mixtures.

the lag phase preceding the autoactivation of procollagenase in culture medium subactivated by trypsin, indicating that they contained an activator of procollagenase (Vaes, 1972b). Further, trypsin-activated culture medium decreased the lag phase preceding the autoactivation of procollagenase in culture fluid that had been subactivated by kallikrein (Fig. 7a) or plasmin (Fig. 7b), suggesting that activation by trypsin, kallikrein and plasmin involved the same endogenous activator of procollagenase.

Assay and properties of the endogenous activator

Previous studies (Vaes, 1972b) on the activation of latent collagenase by trypsin suggested that the endogenous activator is a proteolytic enzyme, distinct from collagenase but also present in a latent trypsin-activatable state in the culture

Fig. 8. Autoactivation of procollagenase in culture fluid subactivated by sodium thiocyanate

Culture fluid was dialysed at 0°C first for 5 h against 3M-NaSCN (▲) or 3M-NaCl (●) and then extensively against the solvent (see legend of Table 1). Dialysed fluid was further preincubated at 25°C for the indicated time and then assayed for collagenase activity. Maximum activity of trypsin-activated medium (Δ) was checked after 24 h preincubation.

Table 2. Effect of culture fluids activated by various agents on the activation of procollagenase in culture fluids sub-activated by trypsin

Culture fluids were activated by one of the activators (see column 1) and the activators were then blocked by an appropriate treatment (column 2). Samples (see column 3) of each activated fluid were added to 1 ml of culture fluid subactivated by trypsin (see under 'Methods'). Collagenase activity of each mixture was then assayed (30 min of incubation) after increasing time of preincubation at 25°C. Results (column 4) are expressed as the time ($t_{\frac{1}{2}}$) after which the collagenase activity of the mixture has reached half of its maximum activity.

Activator	Blocking of activator	Activated fluid added (ml)	$t_{\frac{1}{2}}$ (min)
Lysosomes (700 µg of proteins/ml)	pH adjusted to 7.7	0	75
		0.25	35
Kallikrein (730 µg/ml)	4.5 mM-Dip-F	0	>180
		0.04	155
		0.25	8
Plasmin (180 µg/ml)	2.2 mM-Dip-F	0	165
		0.04	110
		0.25	5
NaSCN (3 M)	Dialysis against buffer (see Table 1)	0	113
		0.04	52
		0.30	3

Fig. 9. Influence of the concentration of endogenous activator on the autoactivation of procollagenase in subactivated medium

(a) Culture fluid was subactivated by a 2 min treatment at 25°C with 1 µg of trypsin/ml. Trypsin was blocked by the addition of 40 µg of soya-bean inhibitor/ml; 2.5 mM-Dip-F was then added to delay the autoactivation. Samples (2 ml) of this subactivated medium were mixed with 0 (▲), 50 (□), 100 (Δ), 150 (○) or 200 (●) µl of trypsin-activated medium, adjusted at a final volume of 2.2 ml with unconditioned culture medium and preincubated at 25°C for the time indicated. Collagenase was then assayed (30 min incubation) in these mixtures. Maximum collagenase activity (about 500 c.p.m.) corresponded to 9.5 units/ml. In (b) the time required for half-maximal activation ($t_{\frac{1}{2}}$) of the subactivated culture fluid has been plotted in relation to the amount of trypsin-activated medium (i.e. of endogenous activator) added.

Table 3. *Influence of various agents on the rate of activation of collagenase in subactivated culture fluid*

Culture media were subactivated (see under 'Methods') by a 2 min treatment at 25°C with 1 µg of trypsin/ml. The action of trypsin was blocked by the addition of either excess of soya-bean trypsin inhibitor or 2 mM-Dip-F. These media were preincubated for various lengths of time at 25°C in the presence of each agent or, in the controls, of the same volume of 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂, after which collagenase was assayed. All the results are expressed as the time ($t_{\frac{1}{2}}$) to reach half-maximum collagenase activity as a percentage of their Tris/CaCl₂ controls; $t_{\frac{1}{2}}$ of the controls varied from one experiment to the other between 28 and 80 min. It was verified that at the concentration used none of the agents tested exerted any significant effect on the collagenase of fully activated media. The agents were dissolved in 0.05 M-Tris/HCl buffer, pH 7.5, containing 2 mM-CaCl₂, except for Dip-F and phenylmethanesulphonyl fluoride, which were dissolved in propan-2-ol, which was 250 mM in the preincubation medium.

Agent	Concentration	$t_{\frac{1}{2}}$ (% of control)
Dip-F	2 mM	500
Phenylmethanesulphonyl fluoride	1 mM	240
Propan-2-ol	250 mM	280
Tos-Lys-CH ₂ Cl	0.2 mM	83
	1.0 mM	86
Egg-white trypsin inhibitor	0.2 mg/ml	117
	1 mg/ml	107
Trasylol	0.06 mg/ml	118
	0.30 mg/ml	118
Serum (human)	2% (v/v)	154
	4% (v/v)	247
Albumin (bovine serum)	1.5 mg/ml	171
	3 mg/ml	149
Casein	2.5 mg/ml	>300
4-Hydroxymercuribenzoate	0.1 mM	22
	0.5 mM	22
N-Ethylmaleimide	1 mM	11
	5 mM	11
Iodoacetate	10 mM	100
Cysteine	0.5 mM	164
	1 mM	245

medium. To allow further characterization of that endogenous activator, it was necessary to develop a quantitative assay for it (Fig. 9). A linear inverse relation was found between the lag time preceding the activation of procollagenase in subactivated culture fluid (measured by the time to reach half-maximal collagenase activity) and the concentration of activator added to that fluid (in the form of a trypsin-activated culture medium). This relation can

Fig. 10. *Effect of culture medium activated by kallikrein, plasmin or trypsin on procollagenase of untreated medium*

Culture fluid was activated by kallikrein (0.73 mg/ml), plasmin (0.18 mg/ml) or trypsin (4 µg/ml) and the activators were blocked by the addition of an excess of Dip-F. Then 1 ml of untreated culture fluid was mixed with 0.25 ml of plasmin- (●) or trypsin- (■) activated medium, or with 0.375 ml of kallikrein- (▲) activated medium. These mixtures were preincubated at 25°C for the times given before the collagenase assays (30 min incubation). In the controls, heat-inactivated medium treated in the same way with either kallikrein (△) or plasmin (○) was used.

be used to measure the amount of activator present in culture media or in purified fractions.

By using this assay technique, the influence of proteinase inhibitors and of other agents on the endogenous activator has been studied (Table 3). Among the proteinase inhibitors tested (Dip-F, phenylmethanesulphonyl fluoride, Tos-Lys-CH₂Cl, egg-white trypsin inhibitor and Trasylol), only Dip-F exerted a slight inhibition on the activator. Propan-2-ol, the solvent used for Dip-F and phenylmethanesulphonyl fluoride, retarded by an unknown mechanism the autoactivation of subactivated culture fluid. More interestingly, the activation of procollagenase in subactivated culture fluids was markedly accelerated by the thiol-blocking agents *N*-ethylmaleimide and *p*-chloromercuribenzoate and retarded by cysteine. Iodoacetate had no effect.

Unlike culture fluids activated by trypsin, which do not activate the procollagenase of untreated culture medium (see Vaes, 1972*b*, and Fig. 10), culture fluids activated by kallikrein or plasmin activated the procollagenase of these media (Fig. 10). This activation cannot be ascribed to residual kallikrein or plasmin activity, because heat-inactivated medium or buffer treated in the same way with kallikrein or plasmin did not activate untreated medium.

Discussion

Since its initial demonstration in culture fluids surrounding mouse bone or skin explants (Vaes, 1971, 1972a), a trypsin-activatable latent precursor of neutral collagenase has been found to be released by several cells or tissues: human polymorphonuclear leucocytes (Kruze & Wojtecka, 1972; Oronsky *et al.*, 1973); fibroblasts from rabbit cornea (Hook *et al.*, 1973), human skin (Bauer *et al.*, 1975), rabbit synovium (Harris *et al.*, 1975) or bovine gingiva (Birkedal-Hansen *et al.*, 1976a); rabbit alveolar macrophages (Birkedal-Hansen *et al.*, 1976c; Horwitz & Crystal, 1976); rat uterus (Woessner, 1975); human synoviocytes (Dayer *et al.*, 1976). A similar latent collagenase was found independently in extracts of tadpole tail-fin tissue; it was, however, not trypsin-activatable, but it could be activated by incubation with tail-fin culture medium (Harper *et al.*, 1971).

The possibility exists that some of these observations are dealing not with a 'procollagenase', but with what could be called a 'meta-collagenase', i.e. with a reversible enzyme-inhibitor complex formed between collagenase and a trypsin-degradable extracellular inhibitor subsequently to the secretion of collagenase and to its action on its substrate. Collagenase can indeed be inhibited by serum α_2 -macroglobulin (Eisen *et al.*, 1971; Werb *et al.*, 1974) or by other serum (Woolley *et al.*, 1975, 1976) or tissue (Bauer *et al.*, 1975) inhibitors, and active collagenase has been recovered after certain treatments of inhibited enzyme (Eisen *et al.*, 1971; Nagai, 1973).

However, it appeared more probable, for reasons discussed in detail elsewhere (Vaes, 1972a; Vaes & Eeckhout, 1975a), that the latent collagenase recovered from bone culture fluids represents an inactive precursor or procollagenase that is secreted as such by the cell and is activated in the extracellular spaces by a limited proteolysis of its molecule to give collagenase and one or several additional peptides. Whether these components are linked inside the procollagenase molecule through covalent bonds (as in a typical zymogen) or through non-covalent bonds (as in most enzyme-inhibitor complexes) is of less importance as far as the physiological function of procollagenase is concerned. This function will indeed depend primarily on the activation of the precursor, and it is thus of fundamental importance to identify the agents responsible for that activation, as their action may indeed be a key factor in the regulation of collagen degradation. Moreover, even if latent collagenase was a 'meta-enzyme' instead of a proenzyme, the possibility that it could be re-activated by some proteolytic agents might also have physiological importance.

Two types of potential proteolytic activators that could have physiological significance were investi-

gated: first, lysosomal acid proteinases, which are released extracellularly together with collagenase in several processes involving connective-tissue degradation (see Woessner, 1968; Dingle, 1969; Reynolds, 1969; Vaes, 1969, 1977); secondly, neutral proteinases such as plasmin, thrombin and kallikrein, which are circulating in the plasma as inactive zymogens and whose activation in vascular or extravascular spaces, occurring under special circumstances (blood coagulation, fibrinolysis, inflammation), could be involved in connective-tissue degradation.

In a first series of experiments, the observation of Vaes (1972a) that purified rat liver lysosomes can activate the procollagenase present in culture fluids of mouse bone explants was confirmed. The lysosomal activator is a thiol-dependent acid proteinase with optimum activity at about pH 6.5. Among the purified lysosomal proteinases tested (cathepsin B, C and D and carboxypeptidase B), only cathepsin B activated latent collagenase. However, it cannot be concluded from the present data that cathepsin B is the sole lysosomal activator of procollagenase. Indeed the capacity to activate latent collagenase compared with the cathepsin B activity, as measured on the synthetic substrate α -N-benzoyl-DL-arginine 2-naphthylamide by the method of Barrett (1972), was about ten times higher in purified rat liver lysosomes than in the purified ox spleen cathepsin B preparation used. This means that either the activity of cathepsin B measured by the method of Barrett (1972) may differ considerably from its activity on procollagenase and that cathepsin B could notwithstanding be the only lysosomal activator of procollagenase, or that one or several still unidentified thiol-dependent lysosomal acid proteinases are also able to activate procollagenase. The fact that chromatography of soluble extracts of purified lysosomes on Sephadex G-100 did not show a clear correlation between the activating capacity of the fractions and their activity on the synthetic substrate (Y. Eeckhout, unpublished work) would favour this last view. Whatever it may be, the experimental data show that the extracellular release of the lysosomal proteinases, especially cathepsin B, may initiate collagen degradation by interaction with procollagenase around physiological pH. At acid pH values, Burleigh *et al.* (1974) observed a collagenolytic activity of cathepsin B which was not detected under the conditions of our collagenase assay.

A second series of data shows that plasmin and kallikrein, but not thrombin, are able to activate procollagenase. The concentration of human plasmin (29 μ g/ml) that activated latent collagenase may be considered physiological, as human plasma contains about 200 μ g of plasminogen/ml (Robbins & Summari, 1970). The possible physiological signifi-

cance of the interactions between plasmin or kallikrein and procollagenase has been discussed elsewhere (Vaes & Eeckhout, 1975b).

The observation that thrombin did not activate procollagenase is unlikely to be due to the presence of heparin (150 μ g/ml) in the preincubation mixture. Indeed in the absence of anti-thrombin higher concentrations of heparin do not inhibit the action of thrombin on fibrinogen (Machovich, 1975).

Although the precise molecular mechanism of the activation of mouse bone procollagenase is still not elucidated (this requires a complete purification of procollagenase), a third group of experimental results shows that, as was observed with trypsin (Vaes, 1972b; Vaes & Eeckhout, 1975a), cathepsin B, plasmin and kallikrein activate procollagenase through an indirect mechanism involving the autocatalytic activation of a latent endogenous activator ('proactivator') present together with procollagenase and a latent neutral proteinase (Vaes *et al.*, 1976) in the bone culture fluids. It appeared likely from previous studies (Vaes, 1972b) that the endogenous activator is a proteolytic enzyme, distinct from collagenase, present in a latent form in the culture fluids and activatable by trypsin. The present data further support that view by showing that cathepsin B, plasmin and kallikrein are also able to activate this 'proactivator' and that the action of the latter is favoured by *N*-ethylmaleimide and 4-hydroxymercuribenzoate but inhibited by cysteine, human serum and bovine serum albumin at concentrations that do not influence the activity of collagenase.

Culture medium activated by either plasmin or kallikrein is able to activate the procollagenase of untreated medium, unlike trypsin-activated culture fluids, which do not activate the procollagenase of the culture fluid unless these fluids have been 'subactivated', i.e. have been in limited contact with trypsin, insufficient to elicit any significant collagenase activity from its precursor. A possible interpretation of this observation is that plasmin and kallikrein render the endogenous activator insensitive to an inhibitor which is present (see Fig. 2 in Vaes, 1972b) in the untreated culture fluids but is removed by plasmin or kallikrein as well as by trypsin during the process of 'subactivation', before the activation of the 'proactivator' and of procollagenase.

Dialysis of culture fluid against 3M-sodium thiocyanate results also in the activation of latent collagenase. This effect of sodium thiocyanate cannot be attributed to the dissociation of a complex between α_2 -macroglobulin and collagenase (Abe & Nagai, 1972; Nagai, 1973) because the apparent molecular weights of latent and active collagenase measured on Sephadex G-150 are respectively about 100000 and 80000 (Vaes, 1972a), whereas that of α_2 -macroglobulin is about 800000. Moreover, if the dialysis against sodium thiocyanate is carried out for a

shorter time, procollagenase remains mostly latent when immediately assayed, although collagenase activity appears 'spontaneously' when the fluids are preincubated for some time at 25°C before the assays. Also, culture fluid activated by sodium thiocyanate accelerates the autoactivation of trypsin-subactivated fluid. All these data suggest that sodium thiocyanate has triggered the autoactivation of the latent endogenous activator and that this leads ultimately to the activation of procollagenase.

In some culture fluids procollagenase was found to become activated spontaneously when incubated at 25°C for 24 or 48 h. This spontaneous activation occurred more or less linearly with time, suggesting that in these fluids a small constant amount of an unidentified activator is responsible for that process. Similar observations were made in crude culture medium of fibroblasts from human skin (Bauer *et al.*, 1975) or bovine gingiva (Birkedal-Hansen *et al.*, 1976a,b).

The latency of the endogenous activator could be due to the presence of an inhibitor which was found (Vaes, 1972b, Fig. 2, curve 7) in most untreated culture fluids. The activation of the endogenous latent activator by enzymic or non-enzymic treatments would thus result from the inactivation of that inhibitor by these treatments. The release of that inhibition would thus allow the activation of procollagenase by the endogenous activator.

To purify the endogenous latent activator and to separate it from procollagenase, a semi-quantitative assay of the activator was developed. Several proteinase inhibitors were tested for their influence on the activator, but none was found that completely inhibited the activator. However, some inhibition was observed, by casein, human serum, serum albumin, cysteine and propan-2-ol. The inhibitory effect of human serum could be non-specific in consideration of the inhibition exerted by serum albumin and casein. Interestingly, the thiol-blocking agents *N*-ethylmaleimide and 4-hydroxymercuribenzoate, but not iodoacetate, stimulated the endogenous activator.

Although high specific activities (3800 units/mg of protein) have been obtained for procollagenase by following the method of Gillet *et al.* (1976, 1977), both neutral proteinase and activator were generally still found associated with procollagenase. The reason for this close association remains to be determined.

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