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Identification and Partial Characterization of an Inhibitor of Collagenase from Rabbit Bone

By ANTHONY SELLERS and JOHN J. REYNOLDS
Cell Physiology Department, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

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Bone explants from foetal and newborn rabbits synthesize and release a collagenase inhibitor into culture media. Inhibitor production in the early days of culture is followed first by latent collagenase and subsequently active collagenase in the culture media. A reciprocal relationship exists between the amounts of free inhibitor and latent collagenase in culture media, suggesting strongly that the inhibitor is a component of the latent form of the enzyme. Over 90% of the inhibitory activity of culture media is associated with a fraction of apparent mol.wt. 30000 when determined by gel filtration on Ultrogel AcA 44. The inhibitor blocks the action of rabbit collagenase on both reconstituted collagen fibrils and collagen in solution. It inhibits the action of either active collagenase or latent collagenase activated by 4-aminophenylmercuric acetate. Latent collagenase activated by trypsin is usually much less susceptible to inhibition. The activity of the inhibitor is destroyed by heat, by incubation with either trypsin or chymotrypsin and by 4-aminophenylmercuric acetate. Collagenase activity can be recovered from complexes of enzyme (activated with 4-aminophenylmercuric acetate) with free inhibitor by incubation with either trypsin or 4-aminophenylmercuric acetate, at concentrations similar to those that activate latent collagenase from culture media. The rabbit bone inhibitor does not affect the activity of bacterial collagenase, but blocks the action of collagenases not only from a variety of rabbit tissues but also from other mammalian species.

There is much evidence that specific collagenases are involved in the turnover of collagen in physiological and pathological conditions (Harris & Krane, 1974*a*,*b*,*c*). To understand the mechanisms of collagen degradation, considerable efforts have been made to characterize specific mammalian collagenases and to identify agents which either stimulate or inhibit their secretion (for reviews see Harris & Krane, 1974*a*,*b*,*c*; Harris & Cartwright, 1977). It has become clear during recent years, however, that collagenases exist in both latent and active forms, suggesting that extracellular enzymic activity may be modulated by factors other than those that control synthesis and secretion.

Latent collagenases from a variety of sources (Vaes, 1972; Harper & Gross, 1972; Kruze & Wojtecka, 1972; Bauer *et al.*, 1972; Hook *et al.*, 1973; Bauer *et al.*, 1975; Birkedal-Hansen *et al.*, 1976; Dayer *et al.*, 1976; Horwitz & Crystal, 1976) all yield active collagenases after limited digestion with trypsin. Explanations for the latency of collagenase have envisaged the existence of either proenzymes, which are believed to be secreted by cells and activated extracellularly by proteinases (Vaes, 1972; Kruze & Wojtecka, 1972; Hook *et al.*, 1973; Birkedal-Hansen *et al.*, 1976; Dayer *et al.*, 1976; Horwitz & Crystal, 1976), or enzyme–inhibitor complexes (Bauer *et al.*, 1972, 1975; Nagai, 1973; McCroskery *et al.*, 1975; Woolley *et al.*, 1976). In the latter postulate, active collagenases are thought to be secreted by cells and their extracellular activity is modified subsequently by inhibitors. Inhibitors of collagenase are the serum proteins α_2-macroglobulin (Nagai, 1973; Werb *et al.*, 1974; Birkedal-Hansen *et al.*, 1976) and β_1-anti-collagenase (Woolley *et al.*, 1976); tissue inhibitors of collagenase from human skin cultures (Bauer *et al.*, 1975), a rabbit tumour (McCroskery *et al.*, 1975), and bovine cartilage and aorta (Kuettner *et al.*, 1976) have also been described. The relationships of these inhibitors to latent collagenases, however, has yet to be determined.

We presented evidence (Sellers *et al.*, 1977*b*) that strongly suggests that latent collagenases are complexes between active enzyme and inhibitors synthesized by connective tissues. In the present paper we report in detail the partial characterization and properties of an inhibitor of collagenase synthesized by rabbit bone. Preliminary accounts of parts of this work have appeared previously (Sellers *et al.*, 1977*a*,*b*).

Materials and Methods

Materials

4-Aminophenylmercuric acetate was from Aldrich Chemical Co., Wembley, Middx. HA0 1PY, U.K. Bovine pancreatic trypsin was from Miles–Seravac (Pty.), Stoke Poges, Slough SL2 4LY, Bucks., U.K. Ultrogel AcA 44 was from LKB, South Croydon, Surrey CR2 8YD, U.K. All other materials have been described elsewhere (Werb & Burleigh, 1974; Sellers *et al.*, 1977*b*).

Methods

Culture techniques. Methods for the growth of tissues and cells *in vitro* for the production of collagenase have been described (Werb & Burleigh, 1974; Werb & Reynolds, 1974). Explants of either parietal bones from rabbit foetus at 22–29 days of gestation or the cranial vaults of newborn (1–2 days old) rabbits were cultured in a modified form of BGJ medium (Reynolds, 1976; one bone per dish containing 1.5ml of medium) supplemented with 5% heat-treated rabbit serum (Burroughs Wellcome, Beckenham, Kent, U.K.). This medium contained no active α_2-macroglobulin or other detectable collagenase inhibitors. Since the explants were not vascularized, they contained negligible amounts of serum proteins.

Collagenase assay. Routinely this was by measurement of the release of ^{14}C-labelled peptides from thermally reconstituted trypsin-resistant fibrils of radioactive rat skin collagen (specific radioactivity 14000d.p.m./mg). Incubations were at 35°C in a final volume of 350μl containing enzyme (plus activators or inhibitor preparations where appropriate), Tris/HCl buffer, pH7.6 (15μmol), NaCl (20μmol), $CaCl_2$,-$2H_2O$ (3μmol) and collagen (100μg). One unit of collagenase hydrolyses 1μg of reconstituted fibrils/min at 35°C (Werb & Burleigh, 1974). Latent collagenase was assayed following activation by either trypsin or 4-aminophenylmercuric acetate (see below), although active collagenase was assayed in the absence of these activators.

Collagenase assays were also made with collagen in solution. Incubations were carried out for 24h at 25°C in a final volume of 350μl containing enzyme (plus inhibitor preparations as required), Tris/HCl buffer, pH7.6 (15μmol), NaCl (20μmol), $CaCl_2$,-$2H_2O$ (4μmol), glucose (200μmol; to prevent fibril formation; Terato *et al.*, 1976) and ^{14}C-labelled rat skin collagen (100μg). The reaction products were analysed by electrophoresis on polyacrylamide gels containing (7%) sodium dodecyl sulphate, after denaturation by boiling for 1min with 1% (w/v) sodium dodecyl sulphate (Neville, 1971).

Activation of latent collagenase. (*a*) With trypsin. Culture fluid containing latent enzyme was incubated with bovine pancreatic trypsin (0.2–0.5mg/ml of activation mixture) for 1min at 25°C. After incubation soya-bean trypsin inhibitor [5-fold (w/w) excess] was added before assay for collagenase.

(*b*) With 4-aminophenylmercuric acetate. Stock solutions (10mM) were prepared daily by suspending the solid (35.2mg) in water (up to 5ml) and bringing this into solution with a minimum of 0.5M-NaOH added dropwise. The pH was adjusted to between 10 and 10.5 with HCl (0.1M) and the final volume of the solution was made up to 10ml with water. Samples were added directly to the fibril assay to give a final concentration of 0.5–1mM. Alternatively, 4-aminophenylmercuric acetate was preincubated with enzyme for 2–4h at 35°C, cooled to 4°C and either chromatographed on Ultrogel AcA 44 or dialysed exhaustively against 50mM-Tris/HCl, pH7.6, containing 200mM-NaCl and 5mM-$CaCl_2$ to remove the excess of 4-aminophenylmercuric acetate.

The activated collagenases produced by activation with either trypsin or 4-aminophenylmercuric acetate were judged to be specific collagenases both by their action on collagen in solution at 25°C to form characteristic products and their sensitivity to the chelating agents EDTA and 1,10-phenanthroline.

Assays for collagenase inhibitor. Collagenase inhibitor, either in culture medium or as a 30000-mol.wt. preparation after gel chromatography (see below), was added to the standard fibril assay in the presence of 0.05 unit of active rabbit bone collagenase (in culture medium). One unit of inhibitory activity was defined as the amount of inhibitor preparation required to inhibit by 50% the fibril lysis produced by 2 units of collagenase under the conditions of the standard fibril assay.

Column chromatography. Concentrated culture media (5ml maximum) with collagenase inhibitory activity were chromatographed on a column of Ultrogel AcA 44 (128cm×1.5cm). Samples were eluted in 50mM-Tris/HCl, pH7.6, containing 200mM-NaCl and 5mM-$CaCl_2$ at a flow rate of 20ml/h. The molecular weight of the collagenase inhibitor was calculated by using the method and standards (bovine serum albumin, 68000; ovalbumin, 45000; soya-bean trypsin inhibitor, 25000; cytochrome *c*, 12500) of Andrews (1964).

Results

Production of collagenase and collagenase inhibitor by rabbit bone explants

We established cultures of bone explants from foetal and newborn rabbits as described under 'Methods', and these were maintained for a maximum of 2 weeks with changes of medium usually every second day. After an initial lag varying from 2 to 6 days, latent collagenase was released into the

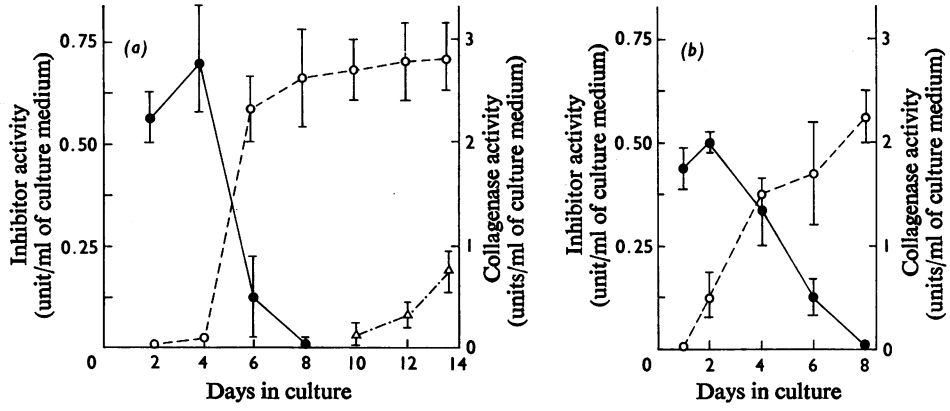

Fig. 1. *Production of collagenase and collagenase inhibitor by explants of foetal and newborn rabbit bone in culture*
Parietal-bone explants from foetal rabbits (*a*) or bone explants from the cranial vault of newborn rabbits (*b*) were maintained in culture as described under 'Methods'. The culture medium was changed every second day and samples of the harvested medium were assayed for latent collagenase (○), active collagenase (△) and collagenase inhibitor (●) (see also under 'Methods'). Results are the mean±S.E.M. of four cultures.

Table 1. *Action of collagenase-inhibitor preparations from rabbit bone on either active or activated collagenase from rabbit bone*
Foetal rabbit bone collagenase (0.035–0.04 unit/assay), either active or activated by initial treatment with 4-aminophenylmercuric acetate or trypsin (see under 'Methods'), was incubated for 20h at 35°C in the standard fibril assay in the presence of approx. 0.025 unit of inhibitor preparations from foetal rabbit bone or 0.02 unit of inhibitor preparations from newborn rabbits. Control incubations contained equivalent volumes of either culture medium or 50mM-Tris/HCl, pH7.6, containing 200mM-NaCl and 5mM-$CaCl_2$. The results are expressed as percentage fibril lysis, ±S.E.M. of four observations in each case. Under the conditions of the assays, fibril lysis was linear with time.

	Inhibitor from foetal rabbit bone			Inhibitor from newborn rabbits		
Type of enzyme	Control	Plus inhibitor	Inhibition (%)	Control	Plus inhibitor	Inhibition (%)
(*a*) With inhibitors in culture media						
Active	36.3±2.3	6.3±0.8	83	36.3±2.3	10.9±2.0	70
Activated with 4-aminophenylmercuric acetate	35.0±3.5	16.3±1.2	53	35.0±3.5	20.2±1.3	42
Activated with trypsin	40.3±2.1	35.0±4.5	12	40.3±2.1	39.1±5.5	3
(*b*) With 30000-mol.wt. inhibitor preparations						
Active	33.0±1.3	6.1±0.1	82	33.0±1.3	12.5±1.5	62
Activated with 4-aminophenylmercuric acetate	38.9±3.2	17.4±3.1	55	38.9±3.2	24.6±3.2	37
Activated with trypsin	38.2±0.5	31.2±0.8	18	38.2±0.5	31.6±4.2	17

medium from explants of both foetal and newborn rabbit (Figs. 1*a* and 1*b*). The latent enzyme could be activated by either trypsin or the thiol-blocking reagent 4-aminophenylmercuric acetate. We have described elsewhere that the most effective reagent for activation is 4-aminophenylmercuric acetate (Sellers *et al.*, 1977*b*). Active collagenase was only released into the culture medium of foetal bone explants towards the end of the culture period, and the amount of collagenase gradually increased as the culture progressed (Fig. 1*a*). Within the period of culture of explants of bone from newborn rabbits, no active collagenase was observed (Fig. 1*b*).

The lag period, before the production of latent collagenase by explants of foetal bone, was characterized by the presence of an inhibitor of collagenase in the culture medium (Fig. 1*a*). As the amount of latent collagenase in the culture medium increased, the inhibitory activity fell to undetectable values. Assays of culture media of bone from newborn rab-

bits harvested before the appearance of latent collagenase again demonstrated the release of a collagenase inhibitor into the culture medium. This inhibitory activity also declined as the amount of latent enzyme in the culture medium increased (Fig. 1*b*).

Bone explants from both sources, either cultured in the presence of cycloheximide (0.2mM) or frozen and thawed three times before explantation, did not release inhibitor or collagenase into the culture medium, strongly suggesting that the inhibitor and enzyme were synthesized by the explants.

Properties of the collagenase inhibitor from rabbit bone

The inhibitor in the culture media from foetal and newborn rabbit bone blocked the action of rabbit bone collagenase, in either the active form or that from latent enzyme activated by 4-aminophenylmercuric acetate (Table 1). The inhibitor was usually less effective on the enzyme activated by trypsin. Pooled culture media from either foetal or newborn rabbit bone explants, which contained inhibitory activity but negligible latent or active enzyme, were chromatographed successively on the same calibrated

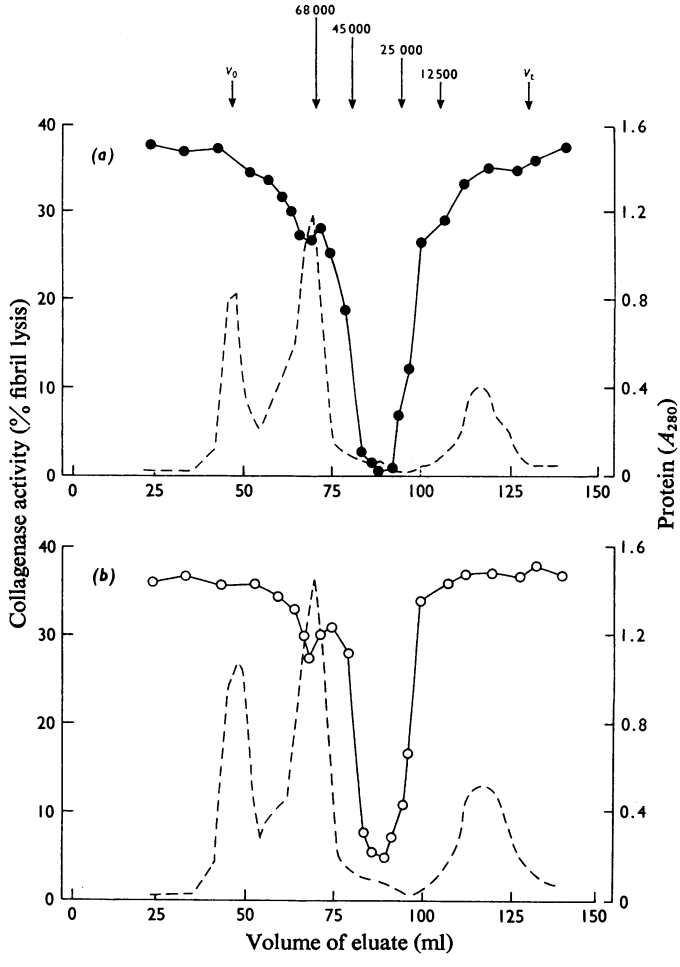

Fig. 2. *Gel chromatography of culture medium containing collagenase inhibitor*
Concentrated culture fluid (5ml) containing collagenase inhibitory activity from either foetal rabbit bone (*a*) or bone from newborn rabbits (*b*) was chromatographed on a calibrated column of Ultrogel AcA 44 as described under 'Methods'. Fractions (approx. 2.5ml) were collected and assayed for inhibitory activity by measuring the percentage fibril lysis produced by 0.05 unit of active rabbit bone collagenase in the presence of samples (100μl) of each fraction in the standard fibril assay. Fractions from foetal rabbit bone (●), fractions from bone of newborn rabbits (○), protein (A_{280} units) ----. V_0, void volume; V_t, total volume of the column.

column of Ultrogel AcA 44. Fractions were assayed for inhibitory activity towards active foetal rabbit bone collagenase. Identical profiles of inhibitory activity were observed for culture medium from foetal rabbit bone (Fig. 2*a*) and from bone of newborn rabbits (Fig. 2*b*). Most of the inhibitory activity (in several experiments greater than 90% of the total) was eluted in a position corresponding to a molecular weight of approx. 30000. A small peak of inhibitory activity was observed in a position corresponding to a molecular weight of approx. 100000. No inhibitory activity was ever observed in fractions eluted in the void volume, excluding the possibility that the inhibitory activity in culture medium was α_2-macroglobulin. Material eluted between 80 and 90ml from each column was pooled, and these preparations of inhibitor (30000-mol.wt. inhibitor preparation) blocked the action of rabbit bone collagenase activated by 4-aminophenylmercuric acetate, but had considerably less effect on latent collagenase activated by trypsin (Table 1).

Equal volumes of different culture media from the same tissue source (first 2 days of culture), which contained different amounts of inhibitory activity, were chromatographed successively on the same column of AcA 44. The ratios of the total inhibitory activities eluted at mol.wt. 30000 (between 80 and 90ml) of each sample were similar to the ratios of the total inhibitory activities of the original samples of culture media before chromatography, again suggesting strongly that the inhibitory activity eluted at mol.wt. 30000 was the major fraction responsible for the inhibitory activity observed in all culture media. This relationship was observed irrespective of whether the source of culture media containing inhibitor was either foetal bone or bone from newborn rabbits.

Subsequent experiments were carried out by using media pooled from cultures of both foetal rabbit bone and the bone from newborn rabbits. The media were harvested before the appearance of latent enzyme, and a semi-purified preparation was then obtained by subsequent gel filtration of the concentrated culture medium (5ml) on a column of Ultrogel AcA 44. The fractions eluted between 80 and 90ml (corresponding to mol.wt. approx. 30000) were pooled.

Incubation of either culture medium containing inhibitor or 30000-mol.wt. inhibitor preparation with trypsin or chymotrypsin (30min at either 25°C or 35°C with 20–100μg/ml) resulted in complete loss of inhibitory activity. The concentration of trypsin required for half-maximal activation of approx. 0.05 unit of latent collagenase in culture medium (10μg/ml) was equal to the concentration of trypsin that destroyed by one-half that amount of inhibitor required to inhibit fully 0.05 unit of active collagenase (Fig. 3). Immersion in a boiling-water bath for 15min destroyed the inhibitor. 4-Aminophenylmercuric acetate (0.5–1.0mM final concentration) protected collagenase from either bone or uterus, activated by 4-aminophenylmercuric acetate, against inhibition by a 30000-mol.wt. inhibitor preparation in the standard fibril assay.

We investigated whether factors that would activate latent rabbit bone collagenase could dissociate complexes formed between activated rabbit bone collagenase and the inhibitor. Enzyme that had previously been activated fully with 4-aminophenylmercuric acetate (4h at 35°C; 1mM-4-aminophenylmercuric acetate: the 4-aminophenylmercuric acetate was subsequently removed by gel filtration on Ultrogel AcA 44) was treated with inhibitor (30000-mol.wt. preparation) at 35°C for 2h. The resulting mixture retained only 10% of the original enzymic activity in a fibril assay. When the reaction mixture was either incubated with trypsin [20–100μg/ml for 30min at 25°C, followed by a 5-fold (w/w) excess of soya-bean trypsin inhibitor] before assay, or if the subsequent fibril assay was carried out in the presence of 4-aminophenylmercuric acetate (0.5–1mM final concentration), then 90–100% of the enzymic activity could be restored. In fibril assays containing 4-chloromercuribenzoate, another thiol-blocking reagent which partially activates latent collagenase (Sellers *et al.*, 1977*b*;

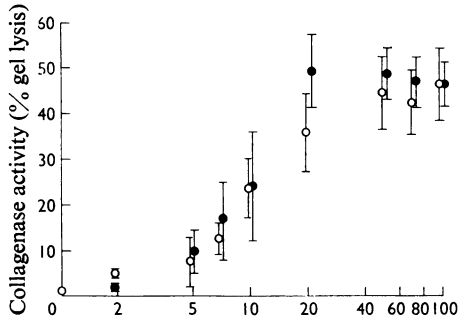

Fig. 3. *Comparison of the activation of latent collagenase and the destruction of collagenase inhibitor by trypsin*
Latent bone collagenase (approx. 0.05 unit) was preincubated for 30min at 25°C with increasing concentrations of trypsin sufficient to activate completely the latent enzyme. Similarly, collagenase inhibitor sufficient to inhibit completely 0.05 unit of active bone collagenase was preincubated with trypsin under the same conditions. Preincubations in each case were terminated by the addition of a 5-fold (w/w) excess of soya-bean trypsin inhibitor. Activation of latent collagenase by trypsin was measured in the standard fibril assay (●). Equal volumes of preincubated inhibitor preparation were assayed for the ability to inhibit 0.05 unit of active collagenase (○). Results are the mean±S.E.M. of three observations in each case.

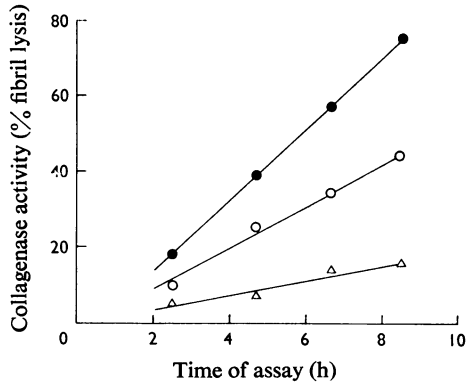

Fig. 4. *Time course of the action of rabbit bone collagenase inhibitor*

Active rabbit bone collagenase (0.15 unit) was incubated either in the absence of inhibitor (●) or in the presence of 0.075 unit (○) or 0.125 unit (△) of inhibitor (30000-mol.wt. preparation) for the times shown in the standard fibril assay. Results are expressed as percentage fibril lysis.

0.5–1.0mM final concentration), only 40–50% of the initial enzymic activity could be recovered. The concentrations of trypsin and 4-aminophenylmercuric acetate that were effective in restoring collagenase activity from collagenase–inhibitor complexes were of the same order as those used to obtain activation of latent collagenase in culture media. Similar results were also obtained by using rabbit uterine enzyme activated by 4-aminophenylmercuric acetate in place of the bone enzyme.

We investigated the time course of inhibition of active rabbit bone collagenase by a 30000-mol.wt. inhibitor preparation (Fig. 4). With a fixed amount of enzyme, we added increasing amounts of inhibitor and measured the percentage of fibril lysis at selected times. Fibril lysis was linear with time throughout the assay period, with or without inhibitor. The action of the inhibitor under these conditions was demonstrable within 2h. That the effect of the inhibitor was fully expressed by this time was indicated by the observation that the percentage inhibition of enzymic activity remained constant during the subsequent period of the assay.

Effect of inhibitor on the activity of collagenase towards collagen in solution at 25°C

Active rabbit bone collagenase was incubated with soluble collagen (see under 'Methods'), and various amounts of 30000-mol.wt. inhibitor preparation were added to incubations sufficient to inhibit the collagenase activity by between 20 and 100% in a standard fibril assay. Control incubations contained an equal volume of 50mM-Tris/HCl, pH7.6, 200mM-NaCl and 5mM-$CaCl_2$. Either culture fluid alone or

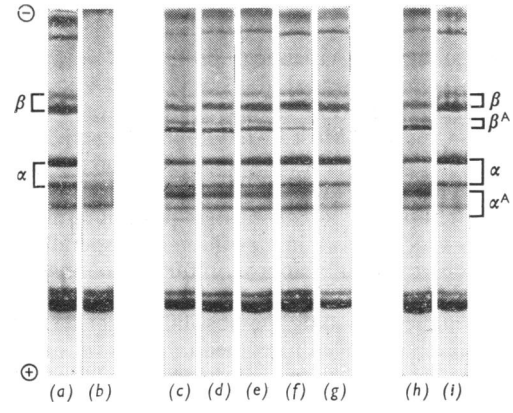

Fig. 5. *Effect of rabbit bone collagenase inhibitor on the action of active rabbit bone collagenase towards collagen in solution*

Incubations were constituted as described in the Experimental and Results sections. Incubation mixtures were denatured with sodium dodecyl sulphate and run on sodium dodecyl sulphate/polyacrylamide gels. The protein bands were stained with Coomassie Brilliant Blue. (*a*) Undegraded collagen with culture medium; (*b*) culture medium alone; (*c*) collagenase (in culture medium) plus column elution buffer; (*d*)–(*g*) collagenase plus increasing amounts of inhibitor (30000-mol.wt. preparation); (*h*) collagenase plus culture medium; (*i*) collagenase plus culture medium containing inhibitor.

culture fluid containing inhibitor sufficient to inhibit the collagenase activity by 80–90% was added to other incubations. The various gel-electrophoresis patterns are shown in Fig. 5. The four bands representing undegraded rat skin collagen (gel *a*) consist of a doublet arising from the single α1- and α2-chains and a doublet corresponding to the β or cross-linked dimers of the α1- and α2-chains. The additional bands on all the gels are proteins of heat-treated rabbit serum, which is a constituent (5%, v/v) of the culture medium (gel *b*). The characteristic products of the cleavage of native collagen by the rabbit bone collagenase are illustrated in Fig. 5, gel (*c*). The β^A and α^A doublets (three-quarter-length fragments) can be seen below the β and α doublets respectively. The bands corresponding to the one-quarter-length fragments (α^B) are not clearly visible. In the presence of increasing amounts of inhibitor the bands of the degradation products gradually disappear (see gels *d*–*g*), until only the bands corresponding to undegraded collagen plus rabbit serum proteins are seen (gel *g*). Similar observations to these were made when culture medium containing inhibitor was used in place of the 30000-mol.wt. inhibitor preparation (see gels *h* and *i*).

Table 2. *Action of inhibitor preparations from rabbit bone on collagenase from other rabbit tissues*
Active or activated collagenases from a variety of rabbit sources (0.035–0.04 unit/assay) were incubated for 20h at 35°C in the standard fibril assay with approx. 0.02 unit of rabbit bone collagenase inhibitor preparation. Control incubations contained an equal volume of either culture medium or 50mM-Tris/HCl, pH7.6, containing 200mM-NaCl and 5mM-$CaCl_2$ (column-elution buffer) in place of inhibitor. The results are expressed as percentage fibril lysis, ±S.E.M. of three observations in each case. Under the conditions of the assay, fibril lysis was linear with time.

Type and source of enzyme	Inhibitor in culture medium			30000-Mol.wt. inhibitor preparation		
	Control	Plus inhibitor	Inhibition (%)	Control	Plus inhibitor	Inhibition (%)
Active						
Skin	29.9±1.3	20.5±0.5	31	32.7±2.5	22.5±1.3	31
4-Aminophenylmercuric acetate-activated						
Skin	31.8±2.5	13.6±1.2	57	32.2±1.3	12.6±2.1	61
Uterus	33.0±1.6	20.0±2.1	39	31.2±2.2	16.9±3.4	46
Synovial cells	28.5±1.3	17.1±0.5	40	32.3±0.6	20.3±0.2	37
Trypsin-activated						
Skin	38.5±3.2	35.2±1.5	9	43.9±3.5	39.1±3.8	11
Uterus	31.5±0.2	27.1±3.1	14	35.2±1.6	29.3±1.5	17
Synovial cells	29.5±2.3	25.1±3.3	15	31.5±2.6	27.1±2.5	14

Table 3. *Action of collagenase-inhibitor preparations from rabbit bone on collagenases from species other than rabbit*
Active or activated collagenases from a variety of sources (0.035–0.04 unit/assay) were incubated for 20h at 35°C in the standard fibril assay with approx. 0.02 unit of rabbit bone collagenase-inhibitor preparation. Control incubations contained an equal volume of either culture medium or 50mM-Tris/HCl, pH7.6, containing 200mM-NaCl and 5mM-$CaCl_2$ (column-elution buffer) in place of inhibitor. The results are expressed as percentage fibril lysis, ±S.E.M. of three observations in each case.

Type and source of enzyme	Inhibitor in culture medium			30000-Mol.wt. inhibitor preparation		
	Control	Plus inhibitor	Inhibition (%)	Control	Plus inhibitor	Inhibition (%)
Active						
Clostridium histolyticum (8ng)	30.5±1.2	31.0±0.2	0	31.3±1.0	31.4±1.9	0
4-Aminophenylmercuric acetate-activated						
Pig synovium	37.0±2.3	15.2±1.8	59	36.5±3.0	13.0±2.5	64
Mouse bone	30.1±0.2	15.6±1.1	48	32.3±2.1	15.3±1.3	47
Trypsin-activated						
Mouse bone	35.3±2.1	32.1±0.7	9	39.3±5.4	33.0±3.2	16

Effect of rabbit bone inhibitor on collagenases from other sources

The ability of either culture fluid containing inhibitor or 30000-mol.wt. inhibitor preparations to inhibit collagenases from various rabbit sources was investigated (Table 2). Both preparations of inhibitor blocked the action of active collagenase from rabbit skin and of collagenase activated by 4-aminophenylmercuric acetate from rabbit skin, uterus and synovial-cell culture media. Enzymes from these sources activated by trypsin were much less suceptible to inhibition, as was found with the enzyme from bone (see Table 1).

We extended these observations by assessing the ability of the rabbit bone inhibitor preparations to block the action of collagenases from sources other than rabbit (Table 3). Specific collagenases from pig synovium and mouse bone produced by activation of the latent enzymes with 4-aminophenylmercuric acetate were inhibited by both inhibitor preparations. Trypsin-activated collagenase from mouse bone was little affected by the inhibitor in comparison with 4-aminophenylmercuric acetate-activated mouse bone enzyme, and active collagenase from *Clostridium histolyticum* was unaffected by inhibitor.

Discussion

The demonstration of an inhibitor of collagenase synthesized by rabbit bone supports the concept that tissue inhibitors may be an important factor in the regulation of collagenase activity. The bone inhibitor differs in apparent molecular weight from other tissue collagenase inhibitors (Bauer *et al.*, 1975; McCroskery *et al.*, 1975; Kuettner *et al.*, 1976) and

from the serum inhibitors of collagenase, α_2-macroglobulin (Nagai, 1973; Werb *et al.*, 1974) and β_1-anti-collagenase (Woolley *et al.*, 1976). The inhibitor blocks the action of collagenase on both reconstituted collagen fibrils and collagen in solution. It has a broad specificity; naturally active collagenases from rabbit bone and skin, collagenases activated by 4-aminophenylmercuric acetate from a variety of rabbit sources, from mouse bone and from pig synovium are all inhibited. These data suggest that similar inhibitors regulate the activity of collagenases in other connective tissues. This view is supported by the identification of inhibitors in the culture media of rabbit skin and uterus explants with properties similar to those of the bone inhibitor (Murphy, *et al.*, 1977).

Collagenases activated by trypsin are usually less susceptible to inhibition by the bone inhibitor. This may be because the enzymic digestion of the latent collagenase molecule by trypsin damages the inhibitor-binding site of the subsequently activated enzyme. Alternatively, trypsin may incompletely degrade the inhibitor, leaving the inhibitor-binding site blocked. The specificity of action of the 30000-mol.wt. inhibitor preparation from gel chromatography does not differ from that of the inhibitory activity in culture medium, implying strongly that the semi-purified preparations represent fully the inhibitory activity initially identified in culture media (Sellers *et al.*, 1977*a*,*b*).

The reciprocal relationship that exists between the amounts of free inhibitor and latent collagenase in the culture media of rabbit bone explants suggests strongly that the inhibitor is a component of the latent enzyme that is found in the later time-period (see Fig. 1). This concept is supported by the observations that the action of the inhibitor is destroyed by trypsin and by 4-aminophenylmercuric acetate under conditions similar to those used to activate latent rabbit bone collagenase. Furthermore, we have shown (Sellers *et al.*, 1977*b*) that a complex between rabbit uterine collagenase activated by 4-aminophenylmercuric acetate and rabbit bone inhibitor has properties similar to those of the latent uterine collagenase found in the culture media of this tissue.

Latent collagenases have mainly been considered as molecules whose extracellular enzymic activity is dependent on the presence of other proteinases for activation (Vaes, 1972; Kruze & Wojtecka, 1972; Birkedal-Hansen *et al.*, 1976; Werb *et al.*, 1977). This view has often been based on the assumption that latent enzyme is secreted from cells. We suggest that active collagenase is secreted by cells and that experimentally observed latent collagenases represent complexes formed extracellularly between active enzyme and the tissue inhibitor. With this hypothesis, active collagenase (and hence increased collagen resorption) could be produced by either increasing enzyme synthesis or decreasing the production of inhibitor.

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