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A Specific Collagenase from Rabbit Fibroblasts in Monolayer Culture

By ZENA WERB and MARY C. BURLEIGH
Tissue Physiology Department, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

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1. Explants of rabbit skin and synovium in tissue culture secreted a specific collagenase into their culture media. Primary cultures of fibroblast-like cells, which were obtained from these tissues and maintained in culture for up to 14 subculture passages, also secreted high activities of a specific collagenase into serum-free culture medium. Secretion of enzyme activity from the cell monolayer was at constant rate for over 100h and continued for up to 8 days in serum-free culture medium. The enzymic activity released was proportional to the number of cells in the monolayer. 2. The fibroblast collagenase was maximally active between pH7 and 8. At 24°C the collagenase decreased the viscosity of collagen in solution by 60%. The collagen molecule was cleaved into three-quarters and one-quarter length fragments as demonstrated by electron microscopy of segment-long-spacing crystallites (measured as native collagen molecules aligned with *N*-termini together along the long axis), and by polyacrylamide-gel electrophoresis of the denatured products. The collagenase hydrolysed insoluble collagen, reconstituted collagen fibrils and gelatin, but had no effect on haemoglobin or Pz-Pro-Leu-Gly-Pro-D-Arg (where Pz = 4-phenylazobenzyloxycarbonyl). 3. The fibroblast collagenase was partially purified by gel filtration and the molecular weight was estimated as 38000. The activity of the partially purified enzyme was stimulated by 4-chloromercuribenzoate, inhibited by EDTA, cysteine, 1,10-phenanthroline and serum, but was unaffected by di-isopropyl phosphorofluoridate, Tos-LysCH_2Cl and pepstatin. 4. Long-term cell cultures originating from rabbit skin or synovium from rabbits with experimentally induced arthritis also secreted specific collagenase. Human fibroblasts released only very small amounts of collagenase.

Collagen is the most abundant connective tissue protein, yet little is known about the mechanism of its turnover in physiological and pathological situations. Gross & Lapière (1962) first reported a specific enzyme capable of degrading native collagen in culture media of tail fins of metamorphosing tadpoles. Since then a number of collagenases have been found in culture fluids of mammalian tissues (reviewed by Lazarus, 1973), including human rheumatoid synovium (Evanson *et al.*, 1968), skin (Eisen *et al.*, 1968; Lazarus & Fullmer, 1969) and mouse bone (Shimizu *et al.*, 1969). Nagai & Hori (1972) directly extracted low collagenolytic activities from human skin and synovial membrane. Collagenases have also been found in synovial fluid (Harris *et al.*, 1969), in the granules of polymorphonuclear leucocytes (Lazarus *et al.*, 1968*a*) and in carcinoma tissue fractions (Harris *et al.*, 1972). None of these sources of enzymes affords a model system for the study of the control of synthesis and release of collagenase at the cellular level. The present study was initiated in an attempt to find such a cellular source of collagenase.

The present paper describes the characterization of a specific collagenase released into the culture medium by viable rabbit fibroblasts and the conditions for its production. This enzyme is compared with the collagenolytic activity produced during the culture of the parent tissues, rabbit synovium and skin. A preliminary report of part of this work has been published (Werb *et al.*, 1973).

Materials

Dulbecco's modification of Eagle's medium (stock concentrate, ×10), crude trypsin [1:250, 2.5% (w/v) in 0.145M-NaCl], Trypan Blue (0.5%, in 0.145M-NaCl) and foetal calf serum were purchased from Flow Laboratories Ltd., Irvine KA12 8NB, U.K. The foetal calf serum was heat-inactivated at 56°C for 30min before use.

Tissue-culture Petri dishes were obtained from Sterilin Ltd., Richmond, Surrey, U.K. Crystamycin (Glaxo Laboratories Ltd., Greenford, Middx. U.K.), and Mycostatin (Nystatin; E. R. Squibb and Sons, Twickenham, Middx. U.K.) were used as antibiotics.

Strangeways Research Laboratory stock New Zealand white rabbits of either sex, male Hooded rats and AKR mice were used. Rabbits were experimentally made arthritic by the method described by Poole *et al.* (1972), and the synovial tissue is referred to throughout as 'arthritic synovium'.

Haemocytometers with counting chambers of 0.2mm depth and modified Fuchs–Rosenthal ruling,

and glass medicine bottles for cell culture (50, 100 and 500ml) were obtained from A. Gallenkamp and Co. Ltd., London E.C.2, U.K. Glutaraldehyde EM [25% (w/v) aq. solution] was purchased from TAAB Laboratories, Reading, Berks. U.K.

Ultrafiltration was carried out in a Diaflo 50 apparatus with a PM-10 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.). Sephadex G-25 and G-100 and Blue Dextran were purchased from Pharmacia (G. B.) Ltd., London W5 5SS, U.K.

Chromatographically purified collagenase from *Clostridium histolyticum* (grade CLSPA, prepared by Worthington Biochemical Corp.) was purchased from Cambrian Chemicals Ltd., Croydon, Surrey CR9 6AG, U.K. Bovine trypsin (2× crystallized), albumin (bovine serum, crystallized), ovalbumin, chymotrypsinogen Tos-LysCH_2Cl (TLCK, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one), cycloheximide, colchicine, insoluble collagen (prepared from bovine achilles tendon), 1,10-phenanthroline and EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] were purchased from Sigma (London) Chemical Co. Ltd, Kingston-on-Thames, Surrey KT2 7BH, U.K.

4-Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Collagenase Chromophor Substrat) made by Fluka A.G. was purchased from Fluorochem Ltd., Glossop, Derbys. SK13 9NU U.K., referred to as Pz-Pro-Leu-Gly-Pro-D-Arg.

PPO (2,5-diphenyloxazole), scintillation grade, was purchased from Packard Instrument Ltd, Caversham, Berks., U.K.

Di-isopropyl phosphorofluoridate (Dip-F) and cytochrome *c* were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and Coomassie Brilliant Blue R250, prepared by Imperial Chemical Industries, was supplied by G.T. Gurr, High Wycombe, Bucks., U.K.

[U-^{14}C]Glycine (90mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Aquacide and Tos-PheCH_2Cl (TPCK, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one) was purchased from Calbiochem Ltd., London WIH 1AS, U.K.

Pepstatin (isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) was the gift of Banyu Pharmaceutical Co. Ltd., Tokyo, Japan.

Ac-$[Ala]_3$-AlaCH_2Cl and Ac-$[Ala]_2$-Pro-AlaCH_2Cl were the gift of Dr J. C. Powers, Georgia Institute of Technology, Atlanta, Ga., U.S.A.

Human, sheep and rabbit sera were the gift of Dr A. J. Barrett. Human synovium and skin were kindly provided by Mr A. Murley and his colleagues of the Orthopaedic Surgery Dept., Addenbrooke's Hospital, Cambridge, U.K. Immunoglobulin G from sheep serum was prepared by $(NH_4)_2SO_4$ precipitation as described by Poole *et al.*, (1972).

Heparin (5000 units/ml) was purchased from Boots Pure Drug Co. Ltd., Nottingham, Notts., U.K.

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

Methods

Culture methods

Cell culture of rabbit synovial fibroblasts. Normal synovia were excised from both sides of the femoral condyles of 6–10-week-old rabbits, washed in several changes of Dulbecco–Eagle's medium buffered with $NaHCO_3$ containing 200 units of penicillin, 200μg of streptomycin and 100 units of mycostatin/ml, then cut into approx. 1mm^3 pieces. Cell strains were established from the explants essentially as described by Hamerman *et al.* (1967) for human synovium. Cells were subcultured after suspension with trypsin (0.125% in Dulbecco–Eagle's medium). The cell number was determined with a haemocytometer. Cell viability was determined by dye exclusion by using Trypan Blue. Cells were 'seeded' in sterile glass flasks or plastic culture dishes, usually at 5×10^4 cells/ml in Dulbecco–Eagle's medium, containing 10% foetal calf serum, and incubated at 37°C in an atmosphere of $CO_2 + O_2 + N_2$ (5:20:75). The medium was changed every 2–3 days until the cell monolayer became confluent. The cells which grew up to confluence and which were passaged were fibroblasts on the basis of their morphology.

Cell culture of other fibroblasts. Primary cell lines from the following tissues were established and passaged in a similar way to rabbit synovial fibroblasts: adult and foetal rabbit skin; arthritic rabbit synovium; mouse skin, synovium and uterus. Mouse 3T3 fibroblasts (gift of Dr. G. A. Dunn, Strangeways Research Laboratory) were cultured in Dulbecco–Eagle's medium with 10% foetal calf serum.

For morphological studies fibroblasts were cultured on glass cover-slips (22mm × 22mm) in plastic Petri dishes, then fixed in 1.25% (w/v) glutaraldehyde in phosphate-buffered saline (0.80% NaCl, 0.02% KH_2PO_4 and 0.12% Na_2HPO_4) for 10min at 4°C. The fixed cells were rinsed thoroughly in water, mounted on a slide over a drop of water, and examined under oil immersion with the Zernicke phase-contrast optics of a Zeiss Photomicroscope II and photographed on 35mm Kodak Panatomic-X film.

Studies of collagenase release. For studies of collagenase production, cell monolayers were rinsed thoroughly in serum-free Dulbecco–Eagle's medium, then cultivated at 37°C in Dulbecco–Eagle's medium in the absence of serum for up to 8 days. The medium was changed at 2–3-day intervals. For studies on the effects of cycloheximide and colchicine on collagenase release, the compounds were dissolved in Dulbecco–Eagle's medium immediately before use, sterilized by passage through a 0.45μm pore-size membrane

filter and diluted to the final concentration with additional Dulbecco–Eagle's medium.

Preparation of collagenase from culture medium. Media were decanted from cultures, adjusted to 50mM-Tris–5mM-$CaCl_2$ with 1M-Tris–HCl buffer, pH7.6, and solid $CaCl_2$, then dialysed against 100mM-Tris–HCl buffer, pH7.6, containing 5mM-$CaCl_2$, and stored at −20°C. Samples were concentrated by ultrafiltration with an Amicon PM-10 membrane or by dialysis against 10% (w/v) Aquacide, as required.

Tissue culture of rabbit skin and synovium. Dorsal skin from 6-week-old rabbits was cultured in Dulbecco–Eagle's medium containing 10mg of bovine serum albumin/ml at 37°C in an atmosphere of $CO_2 + O_2 + N_2$ (5:20:75) essentially as described by Tokoro *et al.* (1972). The culture medium was changed every 2 days for up to 14 days and the culture media were stored at −20°C.

Synovia from normal rabbits and rabbits with experimentally induced arthritis were removed from the sides of the femoral condyles, cut into small pieces, and cultured in a similar manner.

Substrates

Preparation of acid-soluble rat skin collagen labelled with [^{14}C]glycine. Ten rats, aged 40 days at the start of the injection schedule, were each injected intraperitoneally with 48μCi of [^{14}C]glycine in 0.9% NaCl/injection at 96, 72, 60, 48, 36 and 24h before killing by decapitation. Collagen was extracted and purified by a method based on that of Kang *et al.* (1966) with the following modifications. Neutral salt-soluble collagen was extracted by three changes of 0.9% NaCl and discarded; the residue was washed three times in water and the acid-soluble collagen extracted in 0.5M-acetic acid by stirring gently for 2 days. The collagen in the clarified extract was initally precipitated by dialysis against three changes of 5vol. of 5% (w/v) NaCl in 0.1M-acetic acid.

The purified collagen was freeze-dried, stored at −20°C and redissolved in 0.1M-acetic acid as required by stirring overnight at 4°C. The final products had a specific radioactivity of 10140 d.p.m./mg of collagen and contained 13% (w/w) hydroxyproline.

Assay procedures

Assays with reconstituted collagen fibrils. Collagenase activity was determined by the release of radioactive peptides from reconstituted ^{14}C-labelled collagen fibrils essentially as described by Lazarus *et al.* (1968*a*,*b*). Samples (100μl) of the 2mg/ml collagen solution were pipetted into microcentrifuge tubes (Raven Scientific Ltd., Haverhill, Suffolk, U.K.), then incubated at 35°C overnight to obtain reconstituted collagen fibrils. Assays (usually in triplicate) were set up by adding enzyme preparations with 50mM-Tris–HCl buffer, pH7.5 (at 35°C) and 4mM-$CaCl_2$ in a total volume of 200μl, and the fibrils were suspended by shaking. In every experiment, control tubes containing 10μg of trypsin were run to check whether any denatured collagen was present in the preparation. Assays in which the trypsin controls showed a release of more than 5% of the radioactivity above the buffer blanks were discarded. Blanks containing appropriate buffers, media and inhibitors were also included and the pH of each reaction mixture was checked. Samples were incubated at 35°C for up to 24h, then spun in a Gelman Hawksley micro-haematocrit centrifuge at 10000*g* for 10min, and 100μl portions of supernatant were combined with 10ml of scintillation fluid consisting of 300ml of 2-ethoxyethanol, 700ml of toluene and 4g of PPO/litre, and counted for radioactivity in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer to 2% standard deviation.

Total lysis was measured in each experiment by including incubation mixtures with 0.5μg of clostridial collagenase/tube dissolved in 50mM-Tris–HCl buffer, pH7.4, containing 5mM-$CaCl_2$. Total fibril lysis was approximately 2200d.p.m./tube.

One unit of collagenase activity was defined with reconstituted ^{14}C-labelled collagen fibrils as the amount of enzyme hydrolysing 1μg of collagen (3.3pmol)/min at 35°C, i.e. the release of 610d.p.m./h of incubation. Clostridial collagenase (0.55 ± 0.05μg) gave the equivalent lysis of 1 unit of rabbit fibroblast collagenase.

For profiles of enzymic activity as a function of pH, 100μl of 0.1M-sodium acetate, 0.1M-Tris–maleic acid and 0.1M-glycine–NaOH buffers each containing 60mM-$CaCl_2$ were used in the assays. The pH values at 35°C were measured in the complete reaction mixtures and the corresponding buffer controls at the end of the incubation period by centrifuging down remaining fibrils and measuring the pH of the supernatant fluid with a microelectrode.

Assays with collagen in solution. Acid-soluble ^{14}C-labelled collagen was dissolved at 5mg/ml in 0.1M-acetic acid, then dialysed against a large volume of 0.1M-Tris–HCl buffer, pH8.0, containing 0.5M-NaCl and 37.5mM-$CaCl_2$. Aggregates were removed by centrifugation at 20000*g* for 60min in an MSE 65 ultracentrifuge. Reaction mixtures containing final concentrations of 2.5mg of collagen/ml, 20mM-Tris–HCl buffer, pH8, 0.4M-NaCl, 30mM-$CaCl_2$, and enzyme, inhibitors and activators were incubated at 24°C.

For viscometry, measurements were carried out in Ostwald microviscometers [type U.M-4; Camlab (Glass) Ltd., Cambridge CB4 1TH, U.K.] with flow-times for water of 25–28s at 24°C, with a total reaction volume of 2.0ml. The data were expressed as percentages of the initial specific viscosity, $\eta_{sp.}$.

For analysis of reaction products by disc-gel electrophoresis, the mixtures were denatured by

adding urea to give a final concentration of 8M and heating at 50°C for 30min. Electrophoresis of 20μl samples on polyacrylamide gels by the method of Nagai *et al.* (1964) was modified by the use of 150μl of 8M-urea at the top of the gels instead of a sample gel (Daniels *et al.*, 1973). The polyacrylamide gels were fixed in 30% (w/v) trichloroacetic acid for at least 1h, then stained in a mixture of 1ml of aq. 0.5% Coomassie Brilliant Blue R250 and 100ml of 12.5% (w/v) trichloroacetic acid overnight at room temperature; they were photographed on Agfa Copex Ortho film.

Segment-long-spacing crystallites of collagen and the products of reaction with the collagenase were produced by mixing equal volumes of the samples, which had been dialysed overnight against 50mM-acetic acid, and 0.5% (w/v) ATP [desalted on a column of Sephadex G-75 (0.9cm × 20cm) immediately before use as described by Harris *et al.* (1969)]. Specimens were prepared for electron microscopy by negative staining with 10%-satd. ammonium molybdate, and examined in a GEC/AEI EM6B electron microscope at an instrument magnification of × 40000.

Assays with insoluble collagen. Enzymic activity against bovine achilles-tendon collagen and rat skin insoluble collagen was measured in incubation mixtures containing 100mM-Tris–HCl buffer, pH7.6, with 5mM-$CaCl_2$ and the hydroxyproline released was measured as described by Burleigh *et al.* (1974).

Assay of activity on Pz-Pro-Leu-Gly-Pro-D-Arg. Hydrolysis of Pz-Pro-Leu-Gly-Pro-D-Arg was assayed by a modification of the method of Wünsch & Heidrich (1963). Reaction mixtures contained 50mM-Tris–HCl buffer, pH7.5, 0.2M-NaCl, 5mM-$CaCl_2$ and 200μM-Pz-Pro-Leu-Gly-Pro-D-Arg. After the reaction was stopped by the addition of citric acid to 6.7% (w/v), the products were extracted into ethyl acetate instead of benzene and the E_{320} of the upper organic phase was read.

Assays with gelatin. ^{14}C-Labelled gelatin substrate (2mg/ml) was prepared and assays were performed in duplicate essentially as described by Harris & Krane (1972). The reaction mixtures were incubated at 37°C for up to 20h. The material precipitated in 15% (w/v) trichloroacetic acid was removed by centrifugation at 10000*g* for 10min. Samples (100μl) of supernatant were mixed with scintillant and counted for radioactivity as described above for the assay with reconstituted collagen fibrils. In every experiment buffer blanks and control tubes containing 1μg of clostridial collagenase or 3μg of trypsin were included. Both clostridial collagenase and trypsin converted the gelatin into trichloroacetic acid-soluble peptides. Harris & Krane (1972) showed that only peptides of less than 5000 daltons are acid-soluble under these conditions.

Proteinase activity against haemoglobin at pH3.0 and 7.0 was measured as described by Barrett (1970).

Results

Studies on specific collagenase released by rabbit synovium and skin in culture

Rabbit skin, normal synovium and arthritic synovium were cultured in Dulbecco–Eagle's medium containing 10mg of bovine serum albumin/ml. The collagenase activities of the media were determined by the assay with reconstituted fibrils. Results from a typical experiment are shown in Fig. 1. Collagenase activity from skin was greatest from days 4 to 10, then decreased, whereas the activity from normal synovium increased until day 8 and reached a plateau. Enzyme from the arthritic synovium was present from the first 2-day period, and decreased rapidly after day 6, by which time the piece of tissue had disintegrated, but was still viable.

The collagenases from the culture medium of normal and experimentally arthritic rabbit synovial

EXPLANATION OF PLATE I

Plate 1(*a*). *Polyacrylamide-gel electrophoresis of the denatured products of hydrolysis of collagen by the culture media from rabbit synovia*

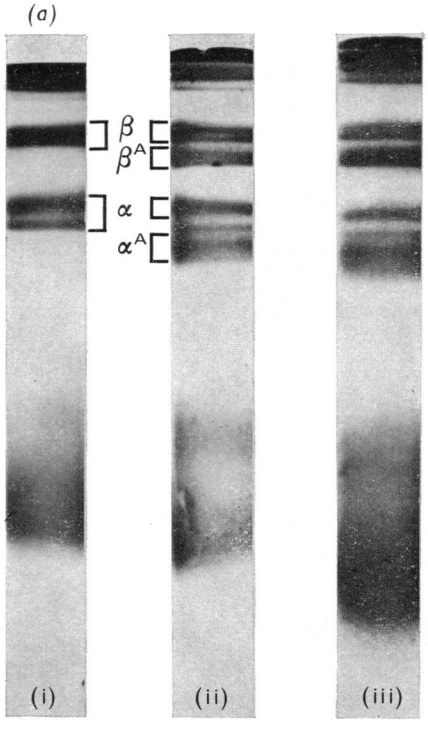
Concentrated culture media were incubated with collagen in solution at 24°C for 20h (see the text). Migration was downwards towards the cathode and the buffer front was at the bottom of the gels. The incubation mixtures contained collagen and (i) control culture medium, (ii) culture medium from normal rabbit synovium and (iii) culture medium from arthritic rabbit synovium. For details of the α and β chains see the text.

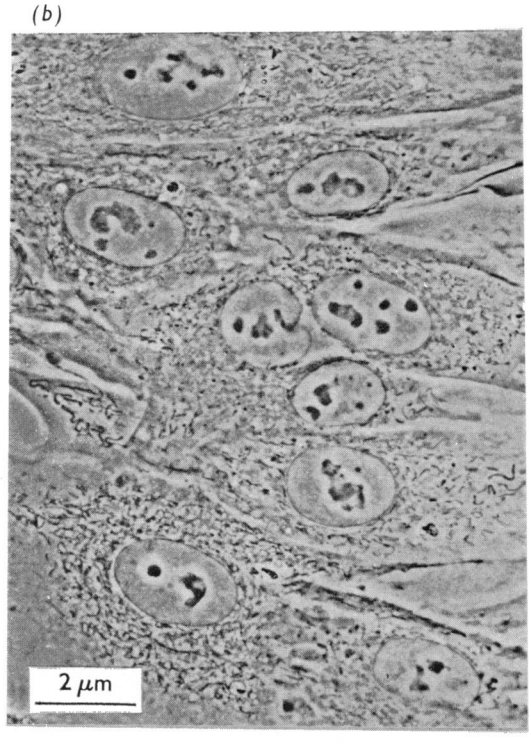
Plate 1(*b*). *Phase-contrast micrograph of a monolayer of rabbit synovial fibroblasts*

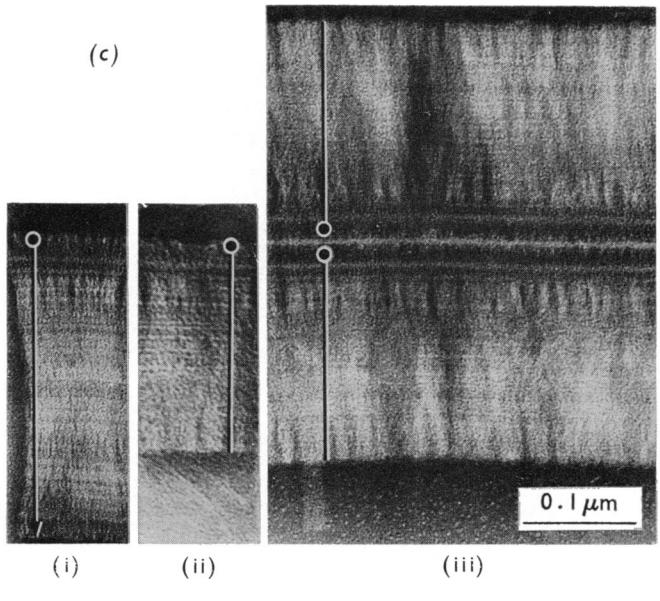
See the text for description. The bar represents 2μm. Magnification ×720.

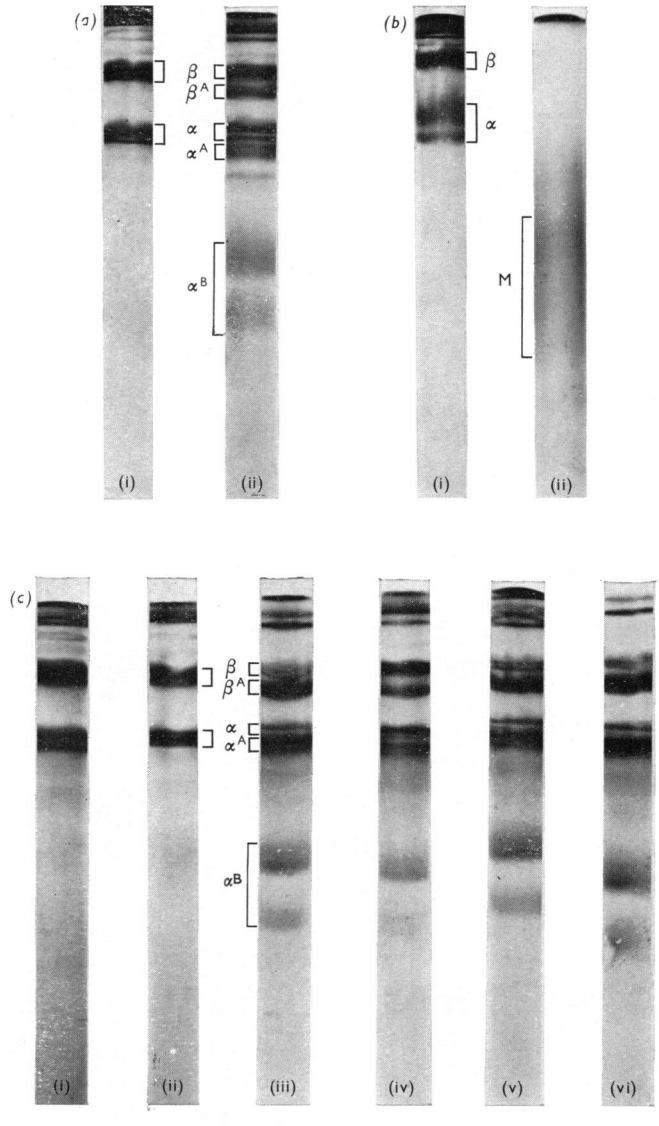
Plate 1(*c*). *Electron microscopy of the reaction products of rabbit synovial-fibroblast collagenase on collagen in solution*

Segment-long-spacing crystallites were prepared from incubation mixtures as described in the Methods and Results sections. Aggregates of (i) intact collagen (0.30μm in length), (ii) A-fragment cleavage product (0.22μm in length), and (iii) a dimer of A-fragment aggregates joined at the *N*-terminal ends are shown. The direction of the arrow indicates the N- to C-terminal arrangement of bands. The bar represents 0.1μm. Magnification ×144000.


(a)
β
β^A
α
α^A
(i)
(ii)
(iii)


(b)
2 μm


(c)
0.1 μm
(i)
(ii)
(iii)



EXPLANATION OF PLATE 2

Gel electrophoresis of the denatured products of reaction of rabbit fibroblast collagenase with collagen in solution

Collagenase was made to react with collagen in solution as described in the text. (*a*) At 24°C: (i) control incubation and (ii) incubation with enzyme. (*b*) At 35°C: (i) control incubation and (ii) incubation with enzyme (M indicates multiple products). (*c*) At 24°C: incubation mixtures containing (iii) enzyme alone, and enzyme plus (i) 10mM-EDTA trisodium salt plus 10mM-cysteine, (iv) 1mM-Dip-F, (v) 0.1mM-Tos-LysCH_2Cl, and (vi) 1mM-4-chloromercuribenzoate. (ii) Control incubation without enzyme. Migration was downwards towards the cathode. For details of the α and β chains see the text.

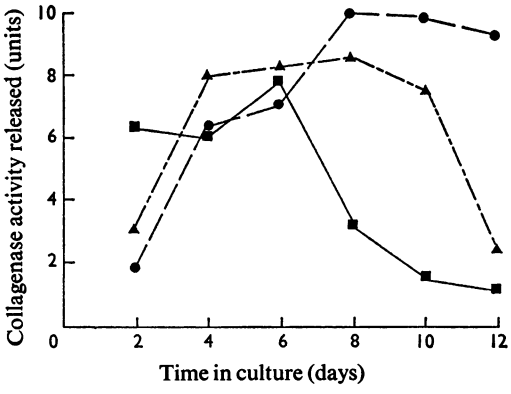


Fig. 1. *Release of collagenase from rabbit tissues in culture*

Tissue (approx. 2cm² areas of rabbit skin, normal synovium and arthritic synovium) in each dish was cultured in 3ml of Dulbecco–Eagle's medium containing 10mg of bovine serum albumin/ml (see the Methods section), with changes of medium every 2 days. Collagenolytic activity was measured in the assay with reconstituted fibrils and expressed as units of collagenase activity/2-day period. Initial wet weights of tissue /dish (mean of four dishes) were 1.03g of arthritic synovium, 0.10g of normal synovium and 0.51g of skin, and wet weights at the end of the experiment (day 12) were 0.65, 0.10 and 0.42g respectively. Collagenolytic activity: ●, normal synovium; ▲, skin; ■, synovium from animals with experimentally induced arthritis. Each point is the mean of triplicate assays on medium from four dishes of tissues. The S.E.M. was less than 15% for each point and is omitted for clarity.

tissue were incubated with collagen in solution at 24°C. Reaction mixtures were denatured by heating in urea and examined by polyacrylamide-disc-gel electrophoresis (Plate 1*a*). In the control incubation mixture containing 10mg of bovine serum albumin/ml of Dulbecco–Eagle's medium, double bands representing the $\alpha 1$ and $\alpha 2$ chains of collagen and the β chains (cross-linked dimers of two $\alpha 1$ chains or one $\alpha 1$ chain and one $\alpha 2$ chain) were seen. Other bands, consisting of α chains with additional cross-links, were also seen. An ill-defined smear of protein was attributable to the albumin present in the reaction mixture. In the reaction mixtures containing culture medium from normal or arthritic synovium a new α^A-doublet appeared below the α chains. These bands corresponded to the large 'A fragment' arising from the A or *N*-terminal end of the α chains. A β^A-doublet appeared below the β chains, corresponding to the cleaved β chains still containing the molecular cross-link near the *N*-terminal. The faster-moving small 'B fragments' (α^B, arising from the *C*-terminal end of both α and β chains) were barely discernible amid the albumin smear. In other experiments where the tissues were cultured in the absence of albumin, the α^B-fragments were clearly seen in the reaction mixtures.

The cleavage of collagen in solution to α^A and β^A fragments by the tissue collagenase was completely inhibited by 10mM-cysteine and/or 10mM-EDTA (trisodium salt) in the reaction mixture, and unaffected by 1mM-Dip-F. Thus the enzyme released into the culture fluids of rabbit synovia and skin was in each case identified as a specific collagenase by the criteria of Gross (1970).

Characterization of rabbit fibroblasts

Cells derived from explants of normal rabbit synovium were cultured in monolayers on glass coverslips for 2 days in Dulbecco–Eagle's medium containing 10% (v/v) foetal calf serum, fixed in glutaraldehyde and examined in the microscope with phase-contrast optics. Typical cells are shown in Plate 1(*b*). The cells had a spindle-shaped fibroblastic morphology. The fibroblasts were large cells with a prominent nucleus with distinct nucleoli, many rod-like mitochondria, phase-lucent and phase-dense granules in the perinuclear zone and an actively ruffled plasma membrane. Many mitotic figures were seen. Cells in confluent monolayers in stationary cultures grew in oriented whorls and appeared smaller in size compared with those in sparse non-confluent exponential-phase cultures.

In the electron microscope the rabbit synovial fibroblasts had many small (100nm) smooth-surfaced vesicles just below the cell surface, and intracellularly a well-developed Golgi apparatus, many mitochondria, stacks of rough endoplasmic reticulum, bundles of microfilaments, a few lysosome-like residual bodies and a few large pinocytic vacuoles.

Cells cultured in serum-free Dulbecco–Eagle's medium for up to 10 days appeared to be of similar morphology. The viable fibroblasts remained adherent to the glass. Fewer mitotic figures were seen, although the cell number doubled in about 50h as compared with about 25h for fibroblasts cultured in serum-supplemented medium.

Characterization of the collagenolytic activity in the culture medium from rabbit synovial fibroblasts

For these studies normal rabbit synovial fibroblasts in 500ml flasks (about 1×10^7 cells/flask) were rinsed with serum-free Dulbecco–Eagle's medium, 20ml of warmed medium was added to each flask, the cultures were equilibrated with $CO_2 + O_2 + N_2$ (5:20:75) and then incubated at 37°C for 3 days. Pooled media were dialysed against 100mM-Tris–HCl buffer, pH7.6, containing 5mM-$CaCl_2$, and concentrated by ultrafiltration to 40% of the original volume.

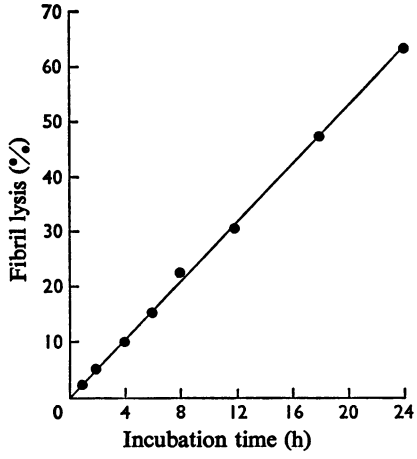


Fig. 2. *Linearity with time of release of radioactive peptides from reconstituted collagen fibrils*

Collagenase (0.1 unit) in 15 μl of concentrated and dialysed culture medium was added to fibrils and incubated at 35°C (see the Methods section). The results are expressed as percentage of the total lysis above the buffer blanks. The total lysis in the assay measured by using clostridial collagenase was 2602 d.p.m., or 1948 d.p.m. above the buffer blanks. The trypsin controls released 81 d.p.m. above the blanks at 24h.

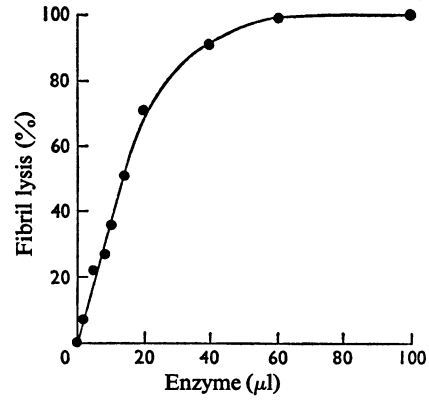


Fig. 3. *Linearity with enzyme concentration of release of radioactive peptides from reconstituted collagen fibrils*

Collagenase (0–100 μl, 27 units/ml) from culture medium was added to each tube, and the mixtures were incubated at 35°C for 4h.

All media from cell lines derived from 17 different rabbits subcultured between one and 14 times were found to have collagenolytic activity.

Collagenolytic activity with reconstituted collagen fibrils

The dialysed and concentrated media from rabbit fibroblast cultures were pooled and examined for collagenolytic activity by use of the assay with reconstituted collagen fibrils (see the Methods section), and the time-course of release of radioactive peptides from ^{14}C-labelled collagen fibrils is shown in Fig. 2. At each time-point tubes with enzyme (0.1 unit of collagenase) were compared with blank tubes containing buffer. Release was linear up to 24h and 64% fibril lysis. In other experiments linearity of release of radioactive peptides with time was found for up to 80% fibril lysis.

The release of radioactive peptides from reconstituted collagen fibrils as a function of the amount of culture medium enzyme in the assay was determined. The results are shown in Fig. 3. The amount of fibril lysis was linear with enzyme concentration to over 70% of total lysis.

The effect of pH on the activity of the collagenase in the culture media of rabbit synovial fibroblasts was determined by using the assay with reconstituted fibrils as described in the Methods section. The experiment was performed in the presence of 20mM-$CaCl_2$ to counteract the metal-ion chelating effect of the buffer constituents. The collagenase had maximum activity between pH7 and 8 with significant residual activity remaining below pH5.5 (Fig. 4). The pH values were determined in the presence of enzyme and collagen, since these solutions buffered in the assay mixtures; buffer blanks were used at each pH. Glycine–NaOH buffers were found to be inhibitory compared with Tris–HCl and Tris–maleic acid buffers at the same pH values. Similar results were obtained when the pH values were measured on complete reaction mixtures after denaturation of the collagen at 50°C for 30min.

Degradation of collagen in solution

Viscometry. The collagenase of culture medium was capable of decreasing the specific viscosity of collagen in solution at 24°C by 60% (Fig. 5). This temperature was selected as it is below the denaturation temperature of collagen or of its products of cleavage by specific collagenase (Evanson *et al.*, 1968). Addition of more enzyme at 24h produced no additional decrease in viscosity. In other experiments the rate of fall of specific viscosity was faster or slower depending on the amount of enzyme added, but the final extent of the decrease was always the same.

Electron microscopy of the products resulting from the action of collagenase on collagen in solution. Reaction mixtures containing 2.5mg of collagen/ml in solution at pH8, and either 5 units of collagenase

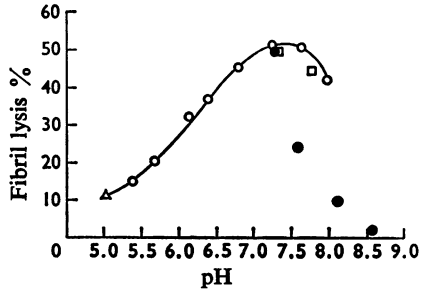


Fig. 4. *pH profile in the radiofibril assay of the collagenase secreted from rabbit synovial fibroblasts*

Collagenase (0.12 unit in 50 μl) was added to each tube, and 100mM buffers containing $CaCl_2$ to give a final concentration of 20mM were added. Blank tubes containing 50 μl of 100mM-Tris–HCl buffer, pH7.6, containing 5mM-$CaCl_2$, instead of enzymes were incubated for each pH. Buffers used: acetate, △; Tris–maleic acid, ○; Tris–HCl, □; glycine–NaOH, ●.

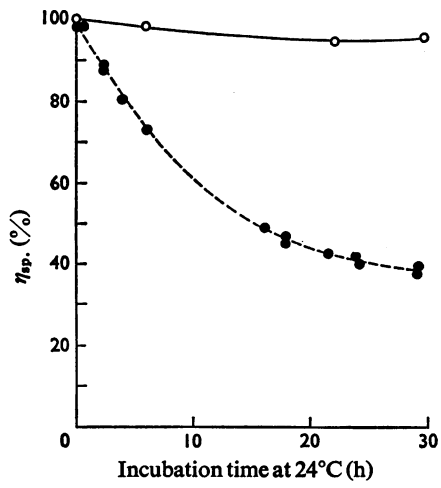


Fig. 5. *Viscometric assay of the collagenase from rabbit synovial fibroblasts on collagen in solution at* 24°C

Viscosity was measured (see the Methods section) of a reaction mixture containing 1ml of collagen in solution (5mg/ml), 400 μl of collagenase (5 units/ml in the assay with reconstituted fibrils) as dialysed and concentrated culture medium, 600 μl of assay buffer (100mM-Tris–HCl, pH8.0, 0.5M-NaCl, 37.5mM-$CaCl_2$) (●). The control reaction mixture contained 400 μl of 100mM-Tris–HCl buffer, pH7.6, containing 5mM-$CaCl_2$ instead of enzyme (○). The results are expressed as percentage of the initial specific viscosity, $\eta_{sp.}$.

(as dialysed and concentrated culture medium), or the same volume of 100mM-Tris–HCl buffer, pH7.6, containing 5mM-$CaCl_2$ as described in the Methods section, were incubated at 24°C in viscometers until the specific viscosity of the enzyme-treated samples had decreased to 40% of the control values. Segment-long-spacing crystallites were prepared and examined in the electron microscope after negative staining. In this procedure the native collagen molecules line up in register along the long axis with the *N*-termini (or A ends) together, and the length of the reaction products may be measured (Gross & Nagai, 1965). As shown in Plate 1(*c*), segment-long-spacing aggregates with intact collagen were seen in control incubations and A fragment aggregates, as judged by aligning the stained bands, were seen in crystallites prepared after reaction with collagenase. The A fragments were 71% [213 ± 1.6nm (± S.E.M., $n = 22$)] of the length of the collagen aggregates. The short B fragments from the *C*-terminal end were difficult to observe and no satisfactory picture was obtained.

Polyacrylamide - gel electrophoresis. Disc - gel - electrophoretic patterns of the products of reaction in solution at 24°C, denatured by heating in urea, are shown in Plate 2(*a*). In control incubation mixtures a doublet arising from the single α1 and α2 chains, a second doublet owing to the β or cross-linked dimers of α1 and α2 chains, and bands corresponding to γ and higher-order aggregates arising from additional cross-links were seen. In the incubation mixture to which the cell-culture medium enzyme had been added and the specific viscosity had decreased 60%, β^A and α^A doublets arising from the denaturation of the A fragments were seen below the β and α doublets respectively; α^B bands arising from the B fragments migrated nearer the buffer front. This pattern was characteristic of the reaction products with specific collagenase (Gross, 1970).

After incubation of the ^{14}C-labelled collagen with culture medium at 24°C, 6% of the radioactivity was soluble in 15% (w/v) trichloroacetic acid whereas there was no trichloroacetic acid-soluble radioactivity in the control mixtures. In contrast, in identical reaction mixtures containing enzyme incubated at 35°C for the same period of time, 72% of the radioactivity was trichloroacetic acid-soluble, and no clearly defined reaction products could be discerned on disc-gel electrophoresis (Plate 2*b*). An ill-defined area of protein staining migrated below the position of the α chains. The controls incubated at 35°C were identical with those at 24°C. At 35°C the cleavage products could be thermally denatured (Evanson *et al.*, 1968) and would then have been susceptible to further hydrolysis by collagenase.

The effects of Dip-F, 4-chloromercuribenzoate, EDTA (trisodium salt) plus cysteine and Tos-LysCH_2Cl are shown in Plate 2(*c*). Dip-F and Tos-LysCH_2Cl had no effect, 4-chloromercuribenzoate slightly activated the collagenase, and EDTA plus cysteine completely inhibited the cleavage of the

collagen. In other experiments 0.1mM-pepstatin was found to have no effect, and 0.1mM-1,10-phenanthroline, 6% (v/v) normal human serum and normal rabbit serum were inhibitory. The inhibition by serum has been shown to be produced by α_2-macroglobulin (Werb, 1973).

Partial purification of rabbit fibroblast collagenase by gel filtration

Serum-free culture media removed from confluent cell cultures up to 6 days after transfer from serum-containing medium were combined, and 1000ml (812 units) was dialysed against 50mM-Tris–HCl buffer, pH7.5, containing 5mM-$CaCl_2$ and 200mM-NaCl, then concentrated to 6ml. The concentrated medium was applied to a column (1.5cm × 90cm; 150ml) of Sephadex G-100 equilibrated with the dialysis buffer, and eluted by downward flow at 20ml/h; 3ml fractions were collected. The collagenase activity was eluted in a single peak between 50 and 64% bed volume. The column was calibrated with Blue Dextran (mol.wt. 2000000), bovine serum albumin (mol.wt. 68000), ovalbumin (mol.wt. 45000), chymotrypsinogen (mol.wt. 25000) and cytochrome *c* (mol.wt. 12400) by the method of Andrews (1965). The collagenase had an apparent molecular weight of about 38000. When fractions at 52–62% bed volume were combined, 90% of the enzyme was recovered (103 units/mg) at an 8.5-fold greater specific activity and was free of non-specific activity on haemoglobin at pH3 and 7. The partially purified collagenase hydrolysed collagen in solution and reconstituted collagen fibrils in a similar manner to the crude culture medium.

Stability. Culture medium stored at −20°C with repeated freeze–thaw cycles retained virtually all its collagenolytic activity after 10 months. Partially purified collagenase could be stored at 4°C for 2 months and at −20°C for at least 6 months with little change in enzymic activity.

Effect of inhibitors and activators on partially purified collagenase

Potential inhibitors were tested on partially purified collagenase in the assay with reconstituted collagen fibrils (see the Methods section) as shown in Table 1. The enzymic activity was inhibited by EDTA, cysteine, EGTA and KCN at millimolar concentrations, and more effectively by 1,10-phenanthroline. The effect of EDTA could be partially reversed by addition of $CaCl_2$. In separate experiments the effect of cysteine was also partially reversed by $CaCl_2$. The chloromethyl ketones, which inactivate cathepsin B1 (Barrett, 1973) and serine proteinases, such as pancreatic elastase (Powers & Tuhy, 1972), had no effect on collagenase. Dip-F, a general inhibitor of serine proteinases, and pepstatin, an inhibitor of carboxyl proteinases, including cathepsin D (Barrett & Dingle, 1972), did not inhibit. Serum inhibited the action of collagenase.

Table 1. *Effect of potential inhibitors on rabbit fibroblast collagenase*

Partially purified collagenase (0.15 unit in 20μl) was used in each assay (see the text) and the inhibitors (80μl) were dissolved in 50mM-Tris–HCl buffer adjusted to pH7.5. Control tubes with the inhibitors and with enzyme replaced by 20μl of eluent buffer were included for each concentration. The assays were incubated for 18h at 35°C (see the Methods section). The values are the mean of at least three assays and are expressed as percentage of the fibril lysis produced by the enzyme alone.

Compound	Concn. in assay (mM)	Activity (% of control)
None		100
EDTA (trisodium salt)	10	0
Cysteine	10	36
EDTA + cysteine	1 + 10	0
EDTA + $CaCl_2$	10 + 20	22
1,10-Phenanthroline	0.001	100
	0.01	27
	0.1	0
	1.0	0
EGTA	10	5
KCN	20	0
Di-isopropyl phosphorofluoridate	1	100
Tos-LysCH_2Cl	0.1	100
Tos-PheCH_2Cl	0.1	84
Ac-[Ala]$_3$-AlaCH_2Cl	0.1	100
Ac-[Ala]$_2$-Pro-AlaCH_2Cl	0.1	100
Pepstatin	0.05	100
Human serum	2% (v/v)	0
Sheep serum	2% (v/v)	0
Rabbit serum	2% (v/v)	0
Foetal calf serum	5% (v/v)	0
Bovine serum albumin	20 mg/ml	100

Potential activators of collagenase were also examined in the assay with reconstituted fibrils (Table 2). Heparin, which had a small stimulating effect at high concentrations, had previously been used in bone culture media to increase yields of collagenase (Vaes, 1972). Thiol-blocking reagents such as 4-chloromercuribenzoate and dithiobisnitrobenzoic acid stimulated the fibroblast collagenase, as has also been shown for granulocyte collagenase (Daniels *et al.*, 1973). 4-Hydroxymercuribenzenesulphonate had little effect. Iodoacetamide had no effect on collagenase activity even when preincubated with the enzyme at 4°C overnight. Colchicine, which has been shown to stimulate release of collagenase from human synovium (Harris & Krane, 1971), had no direct effect.

Table 2. *Effect of potential activators on rabbit collagenase*

Partially purified collagenase (0.15 unit in 20 μl) was used in each assay with reconstituted collagen fibrils. Activators were added as in experiments with inhibitors (Table 1), and reaction mixtures incubated for 18h at 35°C.

Compound	Concn. in assay	Activity (% of control)
None		100
Heparin	0.05mg/ml	87
	0.5mg/ml	119
	5.0mg/ml	153
4-Chloromercuribenzoate	0.0001mM	99
	0.001mM	100
	0.01mM	103
	0.1mM	102
	1.0mM	167
	3.0mM	197
Dithiobisnitrobenzoic acid	0.001mM	122
	0.01mM	125
	0.1mM	137
	1.0mM	153
Iodoacetamide	0.25mM	94
4-Hydroxymercuribenzene-sulphonate	1mM	113
Colchicine	5μg/ml	100

$CaCl_2$ at up to 100mM stimulated collagenase activity. Rabbit fibroblast collagenase dialysed against Ca^{2+}-free 100mM-Tris–HCl buffer, pH7.6, then assayed in the absence of Ca^{2+} in the assay with reconstituted fibrils had variable activities of between 15 and 56% of the activity in the presence of 2mM-$CaCl_2$. At 10mM-$CaCl_2$ the collagenase activity was twice that at 2mM, and remained at this value up to 100mM.

Action of culture-medium collagenase on other substrates

Insoluble collagen. Crude collagenase (4 units) was incubated at 37°C with 4mg of bovine achilles-tendon collagen (as described in the Methods section). The insoluble collagen was hydrolysed by the collagenase, releasing 3.6% of the total hydroxyproline in 3h and 21% in 24h. The collagenase released acid-soluble hydroxyproline-containing peptides from insoluble collagen. A small quantity of peptides which migrated faster than α chains could be seen by disc-gel electrophoresis. These peptides were similar to the products observed after the action of collagenase on tropocollagen in solution at 35°C. The collagenase also hydrolysed insoluble collagen prepared from rat skin under similar conditions.

Gelatin. The activity of crude collagenase and partially purified collagenase against gelatin prepared from ^{14}C-labelled acid-soluble collagen was assayed as described in the Methods section. The collagenase degraded gelatin to pieces soluble in 15% (w/v) trichloroacetic acid. The activity of partially purified collagenase on gelatin was stimulated 50% by 1mM-4-chloromercuribenzoate, unaffected by 1mM-Dip-F and 0.1mM-Tos-LysCH_2Cl, and inhibited by 10mM-cysteine plus 1mM-EDTA and 5% (v/v) normal sheep serum. When the crude culture medium was gel-filtered on Sephadex G-100 the gelatinase activity had exactly the same elution pattern as the collagenolytic activity in the assay with reconstituted collagen fibrils. Hence it was likely that the enzymic activity hydrolysing the gelatin was due to the collagenase.

Pz-Pro-Leu-Gly-Pro-D-Arg. Enzymic activity capable of hydrolysing Pz-Pro-Leu-Gly-Pro-D-Arg was detected in culture medium freshly decanted from monolayer cell cultures. However, this activity was labile to freezing and thawing and could not be detected after storage of crude medium. Since collagenase activity was unaffected under these conditions, and since gel filtration of freshly prepared culture medium separated the peptidase activity from collagenase, it was concluded that this was a separate and non-interfering enzyme similar to the peptidases found previously in culture fluids from tadpole tails and human synovium (Harper & Gross, 1969; Harris & Krane, 1972).

Haemoglobin. A small amount of acid proteinase activity at pH3 was present in the crude culture medium but there was no detectable activity against denatured haemoglobin at pH7. The activity at pH3 was inhibited by pepstatin which had no effect on the collagenolytic activity.

Kinetics of production of specific collagenase by rabbit fibroblasts

Time-course of enzyme release. Four 500ml culture flasks each containing monolayers of about 7×10^6 fibroblasts were rinsed thoroughly in Dulbecco–Eagle's medium; 15ml of medium was then added to each flask, which was gassed with $CO_2 + O_2 + N_2$ (5:20:75) and incubated at 37°C. The medium was decanted at intervals of 6–24h and replaced with fresh medium. At the end of the experiment the medium samples were dialysed against 100mM-Tris–HCl, pH7.5, containing 5mM-$CaCl_2$, concentrated with Aquacide to 0.2vol. and assayed in the assay with reconstituted collagen fibrils. The results are shown in Fig. 6. The cumulative sum of the collagenase activity in the medium increased linearly to 100h, and 0.84 ± 0.08 unit/h was released. In this experiment the cell number increased by less than 25% during the experiment.

In another experiment 14 confluent fibroblast monolayers in 100ml flasks were rinsed in Dulbecco–Eagle's medium, 8ml of medium was added to each,

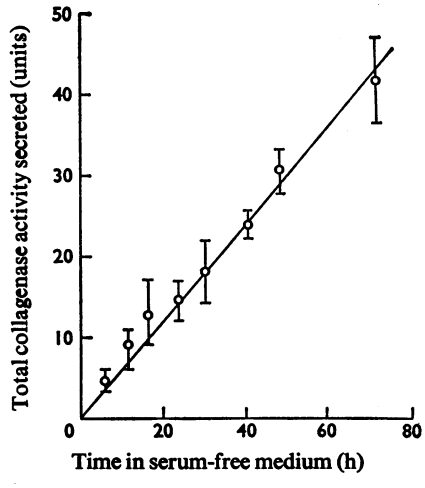


Fig. 6. *Cumulative release with time of collagenase from cultures of rabbit synovial fibroblasts*

Monolayer cultures were incubated in serum-free Dulbecco–Eagle's medium with changes of medium at 6, 12, 18, 24, 30, 41, 49 and 66h after the initial change (see the text for details). The results are plotted as the cumulative enzyme activity (in units of collagenase in the assay with reconstituted fibrils) at each time. The error bars are ± S.E.M.

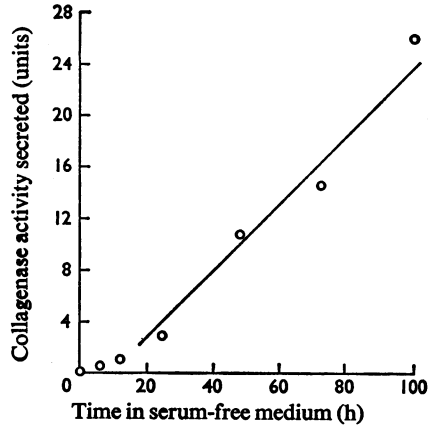


Fig. 7. *Time-course of release of collagenase from rabbit synovial fibroblasts into the serum-free medium*

Collagenase activity accumulation with time in the absence of medium changes was studied. Monolayer cultures were incubated in Dulbecco–Eagle's medium for 0–100h and collagenase activity accumulated in the medium in each period was measured (see the text for details). The line was plotted by the method of least squares ($r = 0.965$, $P < 0.01$).

then pairs of flasks were incubated without changing the medium for up to 100h. At each time-interval the medium was decanted, dialysed and concentrated, and the cell number counted. The cells present in each monolayer increased from 1.8×10^6 at the beginning of the experiment to 3.3×10^6 at 100h. The detectable collagenase activity secreted was linear with time up to 100h (Fig. 7). The deviation from linearity early in the experiment could be accounted for by the increase in cell number during the experiment, by the carry-over of a small amount of serum, adsorbed to the monolayer at the start of the experiment, which inactivated some of the enzyme, or by the inability to measure very low enzymic activities accurately. Collagenase could be detected in the culture medium of rabbit fibroblasts for 10 days, with changes of medium every 2 days. At this time the cell monolayer was fragile, owing to lack of serum, and if foetal calf serum (10%, v/v) was added at the tenth day, collagenase activity could be found in the medium during a second cycle of serum-free medium.

Cell number. The secretion of collagenase as a function of the number of cells in 50mm plastic culture dishes was studied. The exponential-phase cells were placed in serum-free culture medium for 24h and the collagenase activity and cell number determined (Fig. 8). The detectable collagenase activity secreted was linearly related to the number of cells per dish. There was considerable variability in the collagenase activity in the medium for low cell densities, where the measurable enzymic activity was

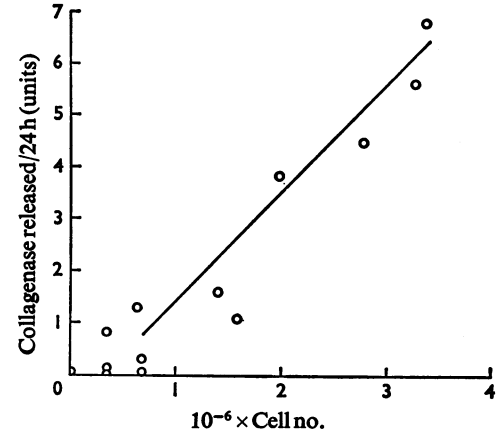


Fig. 8. *Secretion of collagenase as a function of cell number*

Cultures with 0.3×10^6–3.4×10^6 cells were incubated in Dulbecco–Eagle's medium for 24h and the collagenase activity was measured by the assay with reconstituted fibrils (see the text for details). The line was plotted by the method of least squares ($r = 0.957$, $P < 0.01$).

probably decreased by inactivation of the collagenase by factors in the medium.

Number of subculture passages. The secretion of collagenase from rabbit synovial fibroblasts was measured in cultures from the first to the sixth passage after establishment of the primary cell line from explanted synovial tissue. At each weekly subculture, cells were seeded into 100ml flasks and cultured in Dulbecco–Eagle's medium containing 10% foetal calf serum until a confluent monolayer was established. The cultures were then transferred into serum-free medium for 96h with one change of medium after the first 48h. The cell numbers were determined and the media were frozen until the end of the experiment. The collagenase activity present in the medium was essentially the same at each subculture. The mean rate of secretion of collagenase activity was 0.074 ± 0.006 (± S.E.M.) unit/h per 10^6 cells. In independent measurements of collagenase released from fibroblasts it was found that the collagenase secretion remained at about this rate even after the 14 subcultures.

Effect of cycloheximide. Confluent monolayers of fibroblasts were cultured in Dulbecco–Eagle's medium with or without 5μg of cycloheximide/ml for 24 and 48h, and the collagenolytic activity secreted into the medium was measured in the assay with reconstituted collagen fibrils. In the presence of cycloheximide 95% of the cell number compared with controls without the drug remained after 24h and 78% after 48h. Collagenase activity in the medium from cycloheximide-treated cultures was 41 and 16% of control values at 24 and 48h respectively. Cycloheximide had no direct inhibitory effect on collagenase activity in the assays.

Effect of colchicine. Colchicine has been found to stimulate the secretion of collagenase into the culture fluid of primary explants of human rheumatoid synovium (Harris & Krane, 1971). The effect of colchicine on the secretion of collagenase into serum-free culture medium of rabbit synovial fibroblasts was studied. Colchicine (0.5, 1.0, 5.0 and 10.0μg/ml) was added to cultures for 24 and 48h. The colchicine arrested cellular division, resulting in an increase in number of cells in metaphase; however, there was no change in the collagenolytic activity secreted into the medium.

Collagenase secretion from other cell types

Fibroblasts derived from rabbit skin (adult and foetal) and synovium from rabbits with an experimentally induced arthritis also secreted collagenolytic enzymes when cultured in serum-free Dulbecco–Eagle's medium. The collagenase from rabbit skin fibroblasts was found to give specific β^A, α^A and α^B cleavage products from collagen in solution at 24°C, was inhibited by 10mM-EDTA, 10mM-cysteine and by serum, and was activated by 1.0mM-4-chloromercuribenzoate. In contrast, very small amounts of collagenolytic activity were secreted by confluent monolayers of fibroblasts from human skin and synovium. Cell cultures from mouse skin, synovium and uterus could not be maintained longer than one subculture passage because primary mouse fibroblasts are unstable in culture (Paul, 1970). A stable line of mouse cells (3T3) did not release measurable collagenolytic activity into serum-free medium.

Discussion

Enzymes capable of degrading native collagen at neutral pH values have been found in the culture fluids of many human and animal tissues (reviewed by Eisen *et al.*, 1970; Evanson, 1971; Lazarus, 1973), but there is at present no direct evidence for a role of collagenases in tissue remodelling *in vivo* (Gross, 1970). There are no previous reports of collagenases in the medium of cell cultures, and the cellular sources of collagenase produced by tissues in culture have not been established. In order to study the role of this tissue proteinase in remodelling and destruction in connective tissues the present work was designed to establish a cellular source of this enzyme.

Tissues obtained from rabbits, including cornea (Hook *et al.*, 1971), healing wounds (Donoff *et al.*, 1971), and carrageenin granulomas (Perez-Tamayo, 1970) are reported to release specific collagenase into tissue-culture media. In the present work we found that specific collagenases are secreted from rabbit skin and from synovium of normal rabbits and of those with experimentally induced arthritis. Rabbit bone (J. J. Reynolds & M. C. Burleigh, unpublished work) and rabbit ear fibrocartilage (R. M. Hembry, M. C. Burleigh & J. T. Dingle, unpublished work) also release collagenase in tissue culture. Collagenases have been demonstrated directly in rabbit polymorphonuclear leucocytes (Robertson *et al.*, 1972) and carcinoma cells (Harris *et al.*, 1972).

The results described here show for the first time that cultured cells originating from rabbit synovium and skin secrete a specific neutral collagenase, and that the cells retain this differentiated function throughout their life-span in culture. The cells were defined as fibroblasts on the basis of their morphology, growth characteristics and origin from soft connective tissue (Paul, 1970). It seems likely that the cellular sources of the specific collagenase produced during culture of rabbit tissues were the tissue fibroblasts. The number of polymorphonuclear leucocytes present in tissue from normal animals was negligible and could not account for the amount of collagenase, nor the time-course of enzyme release. Since stable lines of fibroblasts were readily obtained as a routine from rabbit synovium and skin, and all cultures

tested released collagenase extracellularly, the rabbit is a useful species in which to study synthesis and release of collagenase. In addition, rabbit collagenases are particularly stable. Cultured human skin and synovial fibroblasts cannot be used at present, since negligible collagenolytic activity can be detected in culture media. Primary mouse fibroblasts stop dividing and become aneuploid after a few cell divisions and thus are not useful for studying collagenase synthesis.

The collagenase produced by rabbit synovium had the same action on collagen as had the enzyme secreted from fibroblasts derived from that tissue, as demonstrated by the cleavage of collagen in solution at 24°C to specific A and B fragments i.e. three-quarters and one-quarter pieces respectively. The enzymes released from the cells and their parent tissues had similar sensitivities to inhibitors and activators.

The collagenase from rabbit cell cultures cleaved soluble collagen into A and B pieces at 24°C. However at 35°C, the initial digestion products were thermally denatured (Evanson *et al.*, 1968) and the collagen was degraded to fragments of less than 5000 daltons as shown by their solubility in 15% (w/v) trichloroacetic acid (Harris & Krane, 1972). It has become increasingly evident that purified collagenase can degrade collagen to small peptides (Nagai *et al.*, 1966; Evanson *et al.*, 1968; Jeffrey & Gross, 1970; Harris 1972; Tokoro *et al.*, 1972) and therefore it must be emphasized that the limited cleavage at 24°C by collagenase serves only as a criterion for identification of these enzymes. At 35°C highly purified collagenase from rabbit synovial fibroblasts readily degrades native collagen in solution, gelatin, insoluble collagen and collagen fibrils to small peptides (Z. Werb & M. C. Burleigh, unpublished work).

Rabbit fibroblast collagenase, partially purified by gel filtration, was inhibited by serum and chelators, unaffected by Dip-F or pepstatin, and stimulated by 4-chloromercuribenzoate. Thus inhibitors of serine, carboxyl and thiol proteinases did not prevent the action of collagenase. Powerful inhibition by metal ion chelators such as EDTA, cysteine, 1,10-phenanthroline and EGTA is consistent with the classification of fibroblast collagenase as a metal proteinase (Hartley, 1960). Studies on the effects of various chelators on purified rabbit skin collagenase indicate that this enzyme may require both Ca^{2+} and Zn^{2+} (M. C. Burleigh, A. C. Warren, G. S. Lazarus & J. J. Reynolds, unpublished work).

The fibroblast collagenase actively hydrolysed native insoluble collagen fibrils, indicating that it could act *in vivo* to degrade mature collagen fibrils. Under conditions of rapid collagen resorption other proteinases and peptidases may participate in the degradation of collagen fragments (Lazarus *et al.*, 1972).

The fibroblast collagenase had significant activity between pH 5 and 6, suggesting that it could be active in localized areas where lysosomal proteinases are active. However, the intracellular localization of this enzyme and its method of secretion have not yet been elucidated in the fibroblasts. In polymorphonuclear leucocytes the collagenase is found in the specific non-lysosomal granules (Robertson *et al.*, 1972), but the contents of these granules are released into the same phagocytic vacuoles, or degranulated extracellularly at the same time as the lysosome-like azurophil granules (Zucker-Franklin & Hirsch, 1964; Bainton, 1973; Henson, 1971). It has been shown that a lysosomal enzyme, cathepsin B1, is capable of attacking collagen by a different mechanism from that of the specific collagenases (Burleigh, 1973; Burleigh *et al.*, 1974). Since cathepsin B1 acts under different conditions the two types of enzymes are unlikely to work simultaneously. The relative contributions of collagenase and cathepsin B1 in collagen degradation *in vivo* remain to be elucidated.

The failure of previous investigators (Evanson *et al.*, 1968) to detect collagenase production from cells could be due to the presence of α_2-macroglobulin, the plasma inhibitor of collagenase, in the serum used in culture media (Werb, 1973; Werb *et. al.*, 1974). Another explanation arises from recent reports that a proenzyme exists for collagenase (Harper *et al.*, 1971; Harper & Gross, 1972; Vaes, 1972); perhaps the failure to detect collagenase activity could be due to the failure to convert procollagenase into collagenase. We have found insignificant collagenase activity secreted from human skin and synovial fibroblasts, and it is possible that rabbit fibroblasts have the ability to convert the proenzyme into active collagenase whereas human cells do not. Studies with antibodies raised to purified collagenase should elucidate the role of the fibroblast in the production of collagenase.

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