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Purification, Characterization and Inhibition of Human Skin Collagenase

By DAVID E. WOOLLEY, ROBERT W. GLANVILLE, DENNIS R. ROBERTS and JOHN M. EVANSON
University Department of Medicine, University Hospital of South Manchester, West Didsbury, Manchester M20 8LR, U.K.

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1. The neutral collagenase released into the culture medium by explants of human skin tissue was purified by ultrafiltration and column chromatography. The final enzyme preparation had a specific activity against thermally reconstituted collagen fibrils of 32 μg of collagen degraded/min per mg of enzyme protein, representing a 266-fold increase over that of the culture medium. Electrophoresis in polyacrylamide disc gels showed it to migrate as a single protein band from which enzyme activity could be eluted. Chromatographic and polyacrylamide-gel-elution experiments provided no evidence for the existence of more than one active collagenase. 2. The molecular weight of the enzyme estimated from gel filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was approx. 60000. The purified collagenase, having a pH optimum of 7.5–8.5, did not hydrolyse the synthetic collagen peptide 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg-OH and had no non-specific proteinase activity when examined against non-collagenous proteins. 3. It attacked undenatured collagen in solution at 25°C, producing the two characteristic products $TC_A(\frac{3}{4})$ and $TC_B(\frac{1}{4})$. Collagen types I, II and III were all cleaved in a similar manner by the enzyme at 25°C, but under similar conditions basement-membrane collagen appeared not to be susceptible to collagenase attack. At 37°C the enzyme attacked gelatin, producing initially three-quarter and one-quarter fragments of the α-chains, which were degraded further at a lower rate. As judged by the release of soluble hydroxyproline peptides and electron microscopy, the purified enzyme degraded insoluble collagen derived from human skin at 37°C, but at a rate much lower than that for reconstituted collagen fibrils. 4. Inhibition of the skin collagenase was obtained with EDTA, 1,10-phenanthroline, cysteine, dithiothreitol and sodium aurothiomaleate. Cartilage proteoglycans did not inhibit the enzyme. The serum proteins α_2-macroglobulin and β_1-anti-collagenase both inhibited the enzyme, but α_1-anti-trypsin did not. 5. The physicochemical and enzymic properties of the skin enzyme are discussed in relation to those of other human collagenases.

The first demonstration of a collagenolytic enzyme derived from human skin was made by Fullmer *et al.* (1966), using the tissue-culture technique adapted from that originally used by Gross & Lapière (1962). The skin enzyme was subsequently characterized as a true neutral collagenase by Eisen *et al.* (1968). Since then, collagenases from a wide variety of human and mammalian tissues have been reported (Harris & Krane, 1974*a*,*b*,*c*), and there is now substantial evidence to support the involvement of these enzymes in collagen catabolism in both normal and pathological connective tissues.

Human skin collagenase has been extensively studied by Eisen, Bauer & Jeffrey (Eisen, 1969; Bauer *et al.*, 1970, 1972*a*,*b*), whose findings showed the enzyme to have the characteristic properties of a true neutral collagenase with respect to its action on soluble collagen. Earlier work (Bauer *et al.*, 1970) suggested that the enzyme existed in two forms, described as 'slow-' and 'fast-moving' according to their behaviour on polyacrylamide-gel electrophoresis, whereas more recently only one enzyme was identifiable after purification by affinity chromatography (Bauer *et al.*, 1971). Such findings have resulted in some uncertainty as to how many forms of active skin enzyme exist.

The role of serum proteins as inhibitors and possible regulators of collagenase activity *in vivo* has been studied by several groups (e.g. Eisen

Abbreviations used: SDS, sodium dodecyl sulphate; Pz, 4-phenylazobenzyloxycarbonyl.

et al., 1970; Bauer *et al.*, 1972*b*; Werb *et al.*, 1974; Berman & Dohlman, 1975; Woolley *et al.*, 1976*a*). The serum anti-proteinase α_2-macroglobulin is a recognized inhibitor of all mammalian collagenases, but the reports of α_1-anti-proteinase (α_1-anti-trypsin) inhibition of collagenase activity have been in doubt (Woolley *et al.*, 1975*c*, 1976*a*).

Since our earlier work on the physicochemical properties of rheumatoid synovial and human skin collagenases (Woolley *et al.*, 1973), in which we found no evidence for the existence of more than one active skin collagenase, we have undertaken the purification of this enzyme and have made a detailed study of its inhibition by human serum proteins. We report here our studies on the properties of the purified skin collagenase with respect to its natural serum inhibitors and its action on various types of soluble collagen, and show that insoluble collagen fibres prepared from human skin are also susceptible to degradation by this enzyme.

Experimental

Dulbecco's modified Eagle's medium, single-strength and without Phenol Red, was purchased from Wellcome Reagents, Beckenham, Kent, U.K. Benzylpenicillin BP (Glaxo Laboratories, Greenford, Middx., U.K.), streptomycin sulphate BP (Dista Products, Liverpool, U.K.) and Nystatin (E. R. Squibb and Sons, Twickenham, Middx., U.K.) were the antibiotics used in the culture system. Disposable tissue-culture flasks (Falcon Plastics) were purchased from Gibco Bio-Cult, Paisley, Scotland, U.K., and disposable Petri dishes from Sterilin Ltd., Richmond, Surrey, U.K. [U-^{14}C]Glycine (90mCi/mmol) and ^{125}I (100mCi/ml) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Hartley-strain guinea pigs were purchased from Animal Suppliers (London) Ltd., Welwyn, Herts., U.K., or were a gift from Dr. J. Lowe, ICI, Alderney Park, Macclesfield, Cheshire, U.K.

For the collagenase assay disposable polypropylene micro-assay tubes were used (Gelman Hawkesley, Lancing, Sussex, U.K.). Bio-Solv solubilizer BBS-3 and butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; Beckman, Glenrothes, Scotland, U.K.] were used with scintillation-grade toluene (BDH, Poole, Dorset, U.K.).

Ultrafiltration of solutions was performed in Amicon apparatus, models 52 or 402 or the model 8-MC micro-ultrafiltration system by using PM-10 membranes (Amicon, High Wycombe, Bucks., U.K.). Sephadex G-200, G-150 and G-100 (superfine), QAE-Sephadex [diethyl-(2-hydroxypropyl)aminoethyl] A-50 and DEAE-Sephadex A-50 were purchased from Pharmacia (GB) Ltd., London W5 5SS, U.K.

Electrophoresis-grade acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Liverpool, U.K. Coomassie Brilliant Blue R250 was purchased from R. A. Lamb, Alperton, Middx., U.K. SDS and the protein standards transferrin, bovine serum albumin, ovalbumin, aldolase, pepsin, carbonic anhydrase, trypsin and haemoglobin were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Pz-Pro-Leu-Gly-Pro-D-Arg-OH, the chromophore collagenase substrate manufactured by Fluka A.G., was purchased from Fluorochem Ltd., Glossop, Derbyshire, U.K. Agarose electrophoresis was performed with Universal plates, manufactured by Corning ACI, Palo Alto, CA, U.S.A. All other chemicals were commercially available A.R.-grade reagents.

Preparations of human and pig proteoglycans were kindly provided by Dr. C. A. McDevitt, Dr. T. Hardingham and Dr. Helen Muir of the Kennedy Institute of Rheumatology, London W6 7DW, U.K.

Tissue culture

Normal skin was obtained at operation from patients undergoing breast surgery. Fragments were washed three times with culture medium and placed in Falcon culture flasks which contained Dulbecco's modified Eagle's medium supplemented with antibiotics as described previously (Woolley *et al.*, 1975*b*). Each flask containing 15–20 explants was gassed with O_2/CO_2 (19:1), sealed, and incubated at 37°C. The culture medium was harvested and replenished daily for 10 days. Each day's medium was mixed with 1M-Tris/HCl buffer, pH7.6, containing 0.25M-$CaCl_2$, to give a final concentration of 20mM-Tris/HCl and 5mM-$CaCl_2$ before storing at −20°C.

Collagen substrates

For the preparation of salt-soluble [^{14}C]glycine-labelled collagen, guinea pigs weighing approx. 225g were each injected intraperitoneally with 200μCi of [^{14}C]glycine in 1.0ml of sterile 0.9% NaCl. Neutral salt-soluble and acetic acid-soluble collagen was extracted from the minced skins as described previously (Woolley *et al.*, 1976*b*), and the collagen preparations were freeze-dried and stored at −20°C until use. Solutions of collagen were prepared by dissolving the collagen in cold potassium phosphate buffer (*I* 0.4), pH7.6, followed by dialysis against 0.4M-NaCl at 4°C. The specific radioactivity of the collagen thus prepared was usually in the range 30000–40000c.p.m./mg of collagen and the purity of the collagen was confirmed by disc electrophoresis in 5% polyacrylamide gels containing SDS.

Soluble type-II cartilage collagen was a gift from Dr. K. Lindberg, Meat Research Institute, Langford, Bristol, U.K., and was prepared from lathyritic

chicks as described previously (Woolley *et al.*, 1975*a*). Soluble type-III collagen was prepared from foetal calf skin as described by Timpl *et al.* (1975). Soluble basement-membrane collagen purified from mouse tumour (Orkin *et al.*, 1977) was kindly provided by Dr. Rupert Timpl, Dr. George Martin and colleagues at the Max-Planck-Institut für Biochemie, Munich, W. Germany.

Insoluble collagen was prepared from human skin by the extraction and purification procedure described by Steven & Jackson (1967).

Assay procedures

Collagenase assay with reconstituted collagen fibrils. Collagenolytic activity was assayed by measuring the release of soluble radioactive products from a pellet of thermally reconstituted [^{14}C]glycine-labelled collagen fibrils by a modification of the method of Nagai *et al.* (1966). Assay conditions were as described previously (Woolley *et al.*, 1976*b*), with each collagen gel containing 60μg of collagen and 2500c.p.m. of ^{14}C.

Assays with collagen in solution and gelatin. Acid-soluble collagen used to determine collagenase properties was dissolved in 0.1M-acetic acid and then dialysed against 50mM-Tris/HCl buffer, pH8.0, containing 0.4M-NaCl and 10mM-$CaCl_2$. Collagen aggregates were removed by centrifugation at 20000*g* for 30min at 2°C. Reaction mixtures for examination by disc gel electrophoresis contained final concentrations of 2.0mg of collagen or gelatin/ml, 50mM-Tris/HCl buffer, pH8.0, 0.2M-NaCl, 10mM-$CaCl_2$ and collagenase. Gelatin was obtained by thermal denaturation of a collagen solution at 60°C for 30min.

Assays with insoluble collagen. Reaction mixtures prepared in plastic micro-assay tubes contained approx. 750μg of insoluble collagen in 200μl of enzyme solution or buffer. Collagenase and trypsin (75μg/ml) were dissolved in 50mM-Tris/HCl buffer, pH7.6, containing 0.17M-NaCl and 10mM-$CaCl_2$, and incubations were carried out at 37°C in triplicate.

After 24h the micro-assay tubes were centrifuged at 20°C for 60min at 20000*g* and a 100μl sample of each supernatant was carefully removed for hydrolysis and subsequent hydroxyproline determination. The collagen pellet was washed twice with Tris/NaCl/$CaCl_2$ buffer and then hydrolysed for hydroxyproline determination.

Assay for non-specific proteinase activity. Initially the casein assay described by Kunitz (1947) was used to detect non-specific neutral proteinase activity in collagenase preparations at various stages of purification. As this method was found to be relatively insensitive to very small amounts of trypsin, a more sensitive proteinase assay system was used involving ^{125}I-labelled bovine serum albumin and haemoglobin. These substrates were labelled with ^{125}I by a modification of the procedure described by Greenwood *et al.* (1963).

Samples of each iodinated substrate containing approx. 100000c.p.m. were dispensed into plastic micro-assay tubes and diluted to 100μl with a 2mg/ml solution of unlabelled protein in 50mM-Tris/HCl, pH7.6, 0.17M-NaCl and 10mM-$CaCl_2$. The enzyme solution to be assayed was added and the total volume adjusted to 200μl with the buffer. After incubation at 37°C for 16h the reactions were terminated by the addition of 100μl of 50% (w/v) trichloroacetic acid. The tubes were left at 2°C for 2h and centrifuged at 20000*g* for 30min at 2°C. Portions (100μl) of the supernatant were transferred to plastic micro-assay tubes and counted for radioactivity in a Wallac Autogamma counter (LKB Produkter, Bromma, Sweden). Assays were performed in triplicate, and buffer controls and three concentrations of trypsin were run with each batch of assays. The ^{125}I-labelled substrates were shown to be sensitive to 0.01μg of trypsin over 16h of incubation, and the response to increasing concentrations of trypsin was shown to be linear up to 14% of the total substrate degraded.

Assay for collagen peptidase activity. Hydrolysis of the synthetic peptide Pz-Pro-Leu-Gly-Pro-D-Arg-OH was assayed by a modification of the method described by Wunsch & Heidrich (1963). Reaction mixtures contained collagenase preparations in 50mM-Tris/HCl buffer (pH8.0)/0.17M-NaCl/10mM-$CaCl_2$ and a final concentration of 0.5mg of Pz-Pro-Leu-Gly-Pro-D-Arg-OH/ml. After incubation at 37°C for 1–6h the reaction was terminated by addition of citric acid (10%, w/v). The products were extracted into ethyl acetate and the A_{440} of this solution was measured.

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

Assay for hydroxyproline. Hydroxyproline was measured by using a technique modified from that of Woessner (1961), with internal hydroxyproline standards. The reagents chloramine-T and *p*-dimethylaminobenzylaldehyde were those described by Grant (1964) and the decolorizer was that described by Prockop & Udenfriend (1960).

Samples were hydrolysed in 6M-HCl for 16h at 110°C, and the solutions were clarified by the addition of the charcoal-resin decolorizer comprising Dowex AG-1 (X8) (20g) and activated charcoal (20g) and filtered. Portions of hydrolysate (not more than 1ml) were neutralized by the sequential addition of NaOH and HCl by using phenolphthalein as indicator. The volume of the neutralized sample was made up to 2.2ml with water and divided into four 0.5ml portions (A, B, C and D). To portions A and B, 0.5ml of half-saturated NaCl was added, and to portions C and D 0.5ml of hydroxyproline standard solutions (2.5μg/ml and 5μg/ml) respec-

tively). Solutions B, C and D were oxidized by the addition of 1ml of chloramine-T solution, and to solution A, the non-oxidized control, 1ml of combined $HClO_4$/*p*-dimethylaminobenzaldehyde reagent [27ml of 70% (w/v) $HClO_4$+73ml of water+100ml of *p*-dimethylaminobenzaldehyde reagent] was added. The solution was left at room temperature (25°C) for exactly 4min, and the oxidation in solutions B, C and D was stopped by adding 1ml of the combined $HClO_4$/*p*-dimethylaminobenzaldehyde reagent. To solution A, 1ml of chloramine-T solution was added. The colour was developed by incubation of all samples in a water bath at 65°C for 12min. The solutions were cooled to room temperature and the A_{560} values read against a reagent blank within 1h. This method was originally suggested by Dr. D. Lee, Oswestry, Salop, U.K.

Disc gel electrophoresis

Enzyme preparations at various stages of the purification procedure were examined by disc gel electrophoresis in 10.5% polyacrylamide gels polymerized with riboflavin (Davis, 1964; Fantes & Furminger, 1967). To permit elution of collagenase activity the enzyme protein was subjected to electrophoresis in polyacrylamide gels at 2°C in 50mM-Tris/glycine buffer, pH8.3, by using a current of 0.8mA/tube for 8h. After electrophoresis one gel was stained with Coomassie Blue and another cut into transverse discs by using a Canalco gel slicer. Each disc was eluted with 300μl of 50mM-Tris/HCl, pH7.6, containing 0.17M-NaCl and 10mM-$CaCl_2$ by gentle shaking overnight at 2°C, and the eluted protein was assayed for collagenase activity.

Electrophoresis in 5% polyacrylamide gels containing SDS (Shapiro *et al.*, 1967; Weber & Osborn, 1969) was used to examine collagen- and gelatin-degradation products. Enzyme reactions were stopped by adjusting to final concentrations of 50mM-sodium phosphate buffer, pH7.0, 4M-urea, 1% 2-mercaptoethanol and heating at 50°C for 30min.

Bromophenol Blue dissolved in the appropriate buffer solution was used as tracking dye. All gels were stained for 3h with 0.2% Coomassie Brilliant Blue R250 dissolved in 45% (v/v) methanol and 9.2% acetic acid. Destaining was done in 45% methanol/9.2% acetic acid overnight followed by three changes of 5% methanol/7.5% acetic acid. The gels were stored in 7.5% acetic acid and photographs were obtained by using Kodak Tri-Xpan sheet film with a Kodak Wratten 12 filter and transmitted incandescent light.

Molecular-weight estimation

The molecular weight of the enzyme was estimated by gel filtration at 4°C in a column (35cm×1.5cm) of Sephadex G-150 (superfine grade). The eluting buffer was 20mM-Tris/HCl, pH8.0, containing 10mM-$CaCl_2$ and 0.2M-NaCl, and 0.66ml fractions were collected. The column was calibrated with the protein standards transferrin (74000), ovalbumin (43000), carbonic anhydrase (29000) and myoglobin (17000), and the void-volume and salt-exclusion values were determined by solutions of Blue Dextran and potassium ferricyanide respectively. Each fraction of the column eluate was assayed for collagenase activity and the A_{280} measured.

The molecular weight of the purified enzyme was also determined in 10% polyacrylamide gels containing SDS (Weber & Osborn, 1969) by using protein standards of known molecular weight for calibration as described previously (Woolley *et al.*, 1975*b*).

Viscometry

Viscometry measurements were made in 1ml-capacity Ostwald viscometers with water flow times at 25°C of 28s. Pure collagenase with and without purified serum proteins was added to the reaction mixture giving final concentrations of 0.84mg of collagen/ml in 50mM-Tris/HCl buffer, pH8.0, 0.4M-NaCl and 10mM-$CaCl_2$.

Electron microscopy

One drop of an insoluble collagen fibre suspension, either before or after exposure to pure collagenase, was placed on a carbon-coated Formvar-treated grid, blotted and stained with 2% (w/v) phosphotungstic acid, pH6.8. The grids were examined in an AEI 801A electron microscope and photographs were usually taken at a direct magnification of ×25000.

Serum-protein fractionation

α_2-Macroglobulin was purified from human serum by an initial precipitation with 33%-satd. $(NH_4)_2SO_4$, followed by column chromatography on Sephadex G-200 and DEAE-Sephadex A-50 (Woolley *et al.*, 1975*c*, 1976*b*). α_1-Anti-trypsin was precipitated from whole serum between 40%- and 60%-satd. $(NH_4)_2SO_4$ and subsequently purified by column chromatography on DEAE-Sephadex A-50 and Sephadex G-150 (superfine grade).

β_1-Anti-collagenase was purified by an initial $(NH_4)_2SO_4$ fractionation between 30 and 45% saturation, the precipitated protein then being redissolved and separated on DEAE-Sephadex A-50 and Sephadex G-150 (superfine grade) as described previously (Woolley *et al.*, 1975*c*, 1976*a*).

Chromatographic purification of human skin collagenase

All procedures described were carried out at 2–4°C. Approx. 1 litre of tissue-culture medium containing active collagenase (usually days 3–8 of culture) was

centrifuged at 20000*g* for 30min and concentrated to about 5ml by using Amicon PM-10 ultrafiltration membranes. This crude enzyme concentrate was fractionated by gel filtration on a Sephadex G-200 column (50cm×2.5cm) by using 20mM-Tris/HCl, pH7.6, containing 0.17M-NaCl and 10mM-$CaCl_2$ as elution buffer. The sample was eluted at 12ml/h and the column effluent was collected in 3ml fractions. Those fractions that contained collagenolytic activity were pooled and concentrated to approx. 2ml by ultrafiltration.

The concentrated enzyme sample was dialysed against 20mM-Tris/HCl buffer, pH8.1, containing 10mM-$CaCl_2$, and applied to a QAE-Sephadex A-50 ion-exchange-resin column (32cm×1.5cm) equilibrated with the dialysis buffer. A gradient (0–0.3M-NaCl) was applied with a buffer flow rate of 15ml/h and each of the 2ml fractions collected was assayed for collagenase activity. Those fractions containing the single peak of enzyme activity, eluted at approx. 0.09M-NaCl, were pooled, concentrated by ultrafiltration and applied to a column (35cm×1.5cm) of Sephadex G-150 (superfine grade). The equilibration and elution buffer was the same as that for the Sephadex G-200 gel-filtration step. The enzyme sample was eluted with a buffer flow rate of 6ml/h, and 1ml fractions were collected and assayed for collagenase activity.

Results

Purification

Skin collagenase was purified from tissue-culture medium containing collagenolytic activity by ultrafiltration and a series of chromatographic steps on Sephadex G-200, QAE-Sephadex A-50 and Sephadex G-150 (superfine grade). The protein separation achieved with this procedure is illustrated in Fig. 1, where the final enzyme preparation migrated as a single band after electrophoresis in polyacrylamide disc gels. The incorporation of an ion-exchange step into the purification procedure was essential primarily for the removal of serum albumin, which migrated as a fast-moving protein on gel electrophoresis and represented the major protein component of the crude enzyme sample. During the purification, only a single peak of elutable enzyme activity was observed for all the chromatographic separations and no evidence was found for the existence of more than one collagenase.

The final specific activity of the purified skin collagenase was 32μg of collagen degraded/min per mg of enzyme protein, which represented a more than 260-fold increase over that of the original enzyme sample (Table 1). The final yield of enzyme was usually small, never exceeding 1mg of protein, and represented about a 10% recovery of the initial enzyme activity. However, the poor stability of the enzyme in its final stages of purification make recovery data difficult to evaluate.

Proteinase and collagen peptidase activities

Purified and crude collagenase samples of known activity against reconstituted collagen fibrils were

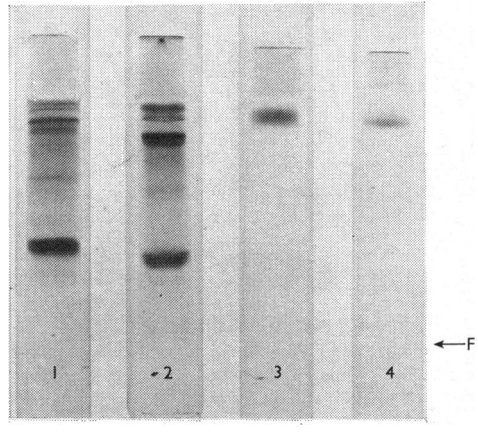


Fig. 1. *Disc-gel-electrophoretic patterns of human skin collagenase preparations from each step of the purification procedure*

Concentrated enzyme preparations (containing approx. 20μg of protein) from tissue-culture medium and the active fractions from Sephadex G-200 and QAE-Sephadex A-50 were applied to 10.5% polyacrylamide gels 1, 2 and 3 respectively. Approx. 10μg of the final enzyme preparation from Sephadex G-150 (superfine grade) was applied to gel 4. F indicates the position of the tracking dye Bromophenol Blue.

Table 1. *Purification of human skin collagenase*

Purification procedure	Specific activity (μg/min per mg)	Increase in specific activity (-fold)	Total protein (mg)	Enzyme recovery (%)
Tissue-culture medium	0.12	(1)	—	—
Ultrafiltration	0.72	6	334	(100)
Sephadex G-200	2.3	19	105	94
QAE-Sephadex A-50	11.3	94	3.0	13
Sephadex G-150 (superfine grade)	32.0	266	0.75	10

Table 2. *Effect of human skin collagenase on various substrates*

Enzyme specific activity is expressed as μg of collagen degraded/min per mg of protein. The amounts of collagenase added to the proteinase assays produced complete lysis of reconstituted ^{14}C-labelled collagen gels (60μg) within 2h. The specific radioactivities of the ^{125}I-labelled bovine serum albumin and ^{125}I-labelled haemoglobin substrates were 500c.p.m./μg of protein. Each assay contained 100000c.p.m. of ^{125}I-labelled protein substrate, and incubations were for 16h at 37°C as described in the Experimental section. All values are means of triplicate assays after subtraction of controls.

	^{14}C-labelled collagen fibrils (specific activity)	Casein (A_{280})	^{125}I-labelled bovine serum albumin (c.p.m.)	^{125}I-labelled haemoglobin (c.p.m.)	Pz-peptide (A_{440})
Crude collagenase (100μg)	0.6	0.05	400	600	0
Purified collagenase (10μg)	22	0	0	0	0
Trypsin (0.5μg)	—	0.164	38000	23400	—
Bacterial collagenase (0.5μg)	—	—	—	—	0.205

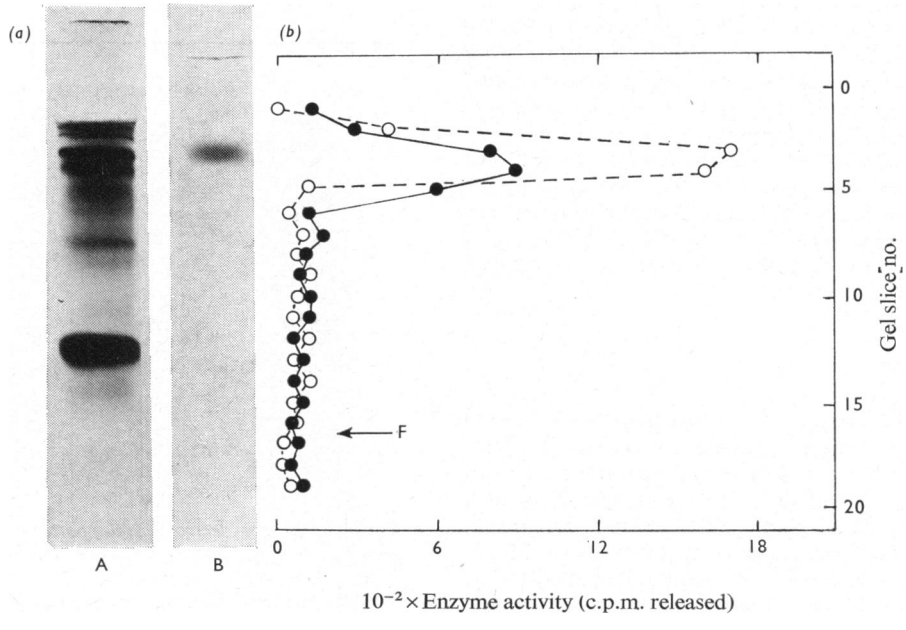


Fig. 2. *Elution of enzyme activity from polyacrylamide disc gels after electrophoresis of purified and crude human skin collagenase*

The crude enzyme sample (A, 25μg) and the purified skin collagenase (B, 10μg) were each applied to 10.5% polyacrylamide gels polymerized with riboflavin and subjected to electrophoresis at 2°C with a current of 0.8mA per tube for 8h. Gels for elution were sliced and the discs were eluted with buffer as described in the Experimental section. The eluates were then assayed for collagenase activity (*b*); ●, A; ○, B. Corresponding non-eluted gels were stained with Coomassie Blue (*a*).

examined against various non-collagenous protein substrates (Table 2). Incubations of the crude enzyme solution with casein, ^{125}I-labelled bovine serum albumin and ^{125}I-labelled haemoglobin revealed a small amount of non-specific proteolytic activity, but when the purified enzyme was exposed to these substrates no degradation was detected even after prolonged incubations. In contrast, trypsin assays run in parallel with the collagenase samples produced significant breakdown of the three protein substrates with relatively small amounts of enzyme.

No collagenase peptidase activity was detected for the two collagenase preparations when incubated with the synthetic collagen peptide Pz-Pro-Leu-Gly-Pro-D-Arg-OH. Bacterial collagenase, on the other hand, known to have very different collagenolytic properties, degraded this synthetic substrate readily. The purified skin collagenase therefore

appears to be specific for collagen, having no detectable activity against the Pz-peptide or non-collagenous substrates. This observation was confirmed by the absence of degradation products from incubations of fibrinogen and transferrin with the purified enzyme when examined by polyacrylamide-disc-gel electrophoresis.

Elution of collagenase from polyacrylamide disc gels

After electrophoresis of the crude and purified skin collagenase samples in polyacrylamide disc gels at alkaline pH and 2°C, enzyme activity was eluted from the sliced gels as indicated in Fig. 2. The activity eluted from the gel containing the purified enzyme was limited to the single stained protein band. A similar but slightly broader single peak of collagenase activity was eluted from the polyacrylamide gel containing the crude enzyme sample. No activity was elutable from the region of the gel containing the faster-moving proteins, the enzyme activity being confined to the upper portion of the gel close to the cathode.

Molecular-weight estimation

The molecular weight of the purified human skin collagenase was estimated by electrophoresis in polyacrylamide gels containing SDS and also by gel filtration on calibrated columns of Sephadex G-150 (superfine grade). Both methods indicated a mol.wt. of approx. 60000.

Electrophoresis of the purified enzyme in an SDS-containing polyacrylamide gel is shown in Fig. 3. This enzyme sample, the same as that used in the elution experiment of Fig. 2, after reduction with 2-mercaptoethanol, migrated mainly as a single protein band of mol.wt. 60000, as judged by the calibration of similar gels with protein standards of known molecular weight. A trace of a faster-moving protein was also observed which had mol.wt. approx. 20000.

Gel-filtration experiments on a calibrated column of Sephadex G-150 (superfine grade) consistently gave molecular-weight values for the purified skin collagenase of approx. 63000 (results not shown). This value was also obtained when the enzyme was eluted with buffers containing 3M-sodium thiocyanate, 1M-NaCl or 0.1% Triton, which suggests that the purified enzyme does not exist as an enzyme–protein complex and cannot be converted into a smaller component by exposure to these dissociating conditions.

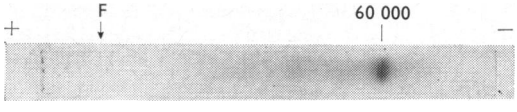


Fig. 3. *Electrophoresis of the purified enzyme sample in 10% polyacrylamide gels containing SDS*
The molecular weight was calculated from gels calibrated with protein standards as described in the Experimental section. F indicates the position of the tracking dye Bromophenol Blue.

Action of purified skin collagenase on various soluble collagens

At 25°C skin collagenase attacks soluble collagen, resulting in a single cleavage of the molecule across the triple-helical structure and the formation of two products called $TC_A(\frac{3}{4})$ and $TC_B(\frac{1}{4})$ (Eisen *et al.*, 1968). This reaction, performed under the optimal conditions of pH 8.0 and 10mM-$CaCl_2$, results in a final decrease in specific viscosity of approx. 58%.

We have examined by disc gel electrophoresis the action of the skin enzyme at 25°C on purified samples of soluble collagen representing types I, II, III and also basement-membrane collagen (Plate 1). Despite differences in the α-chain composition of these collagens, types I, II and III were all cleaved at a region approx. 25% from the *C*-terminus of the collagen molecule to produce the characteristic $\frac{3}{4}:\frac{1}{4}$ products labelled α^A and α^B in Plate 1. However, although the mode of collagenase attack is similar for collagen types I, II and III, differences exist with respect to the relative rates of collagenase attack, type-II cartilage collagen being more resistant than types-I or -III collagen (Woolley *et al.*, 1975*a*; McCroskery *et al.*, 1975).

Of particular note was the observation that mouse basement-membrane collagen was apparently resistant to cleavage by the purified skin enzyme. It is uncertain whether or not this result indicates a complete or partial resistance to collagenase, but parallel studies under similar conditions resulted in complete cleavage of type-I collagen.

Action on gelatin

The products of the reaction at 37°C between the purified skin collagenase and gelatin were examined by disc gel electrophoresis, and Fig. 4 shows the results obtained at various time intervals up to 16h. At 1h the principal products are three-quarter and one-quarter fragments of the α_1- and α_2-chains. By 16h further degradation of these large products has occurred, and, in particular, it is clear that the α_2(I)-chains have been degraded more rapidly than the α_1(I)-chains.

Action on human skin insoluble collagen

When purified insoluble collagen fibres derived from human skin were incubated at 37°C with the purified skin collagenase significant amounts of hydroxyproline were released into solution (Table 3). Incubations of this collagen substrate with trypsin also released a small amount of hydroxyproline, but far less than that with the collagenase. The same

purified collagenase preparation was assayed against reconstituted collagen fibrils for much shorter periods and a value was extrapolated for a 24h incubation (Table 3). This showed that the insoluble collagen substrate was about 20 times more resistant to degradation than was the reconstituted collagen, which contained no cross-links.

Further evidence for collagenase degradation of insoluble collagen fibres was obtained from electron microscopy. Plate 2 shows micrographs of collagen fibres before and after exposure to purified skin collagenase. The control incubations without enzyme showed only intact collagen fibres, whereas those exposed to enzyme revealed extensive degradation, with fibres disintegrated and frayed at the ends. Small fragments of fibres were seen in various stages of dissolution, and these often suggested a helical packing arrangement for the component fibrils.

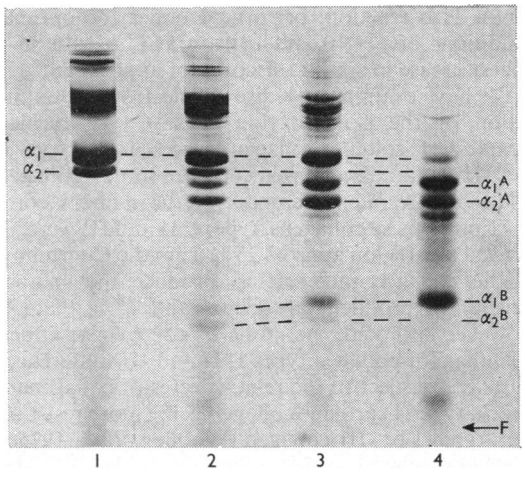


Fig. 4. *Disc gel electrophoresis of gelatin before and after exposure to purified skin collagenase at 37°C*

Portions (30μl) of a reaction mixture (250μl) containing gelatin (500μg) and collagenase (approx. 15μg) were taken at 0, 1, 3 and 16h of incubation and examined in 5% polyacrylamide gels containing SDS (gels 1–4 respectively). Each gel contained approx. 60μg of protein. The $\frac{3}{4}$- and $\frac{1}{4}$-length fragments of the α-chains are labelled α^A and α^B respectively. Note the absence of the α_2-band from gel 3 and α_2^B-band from gel 4. F, position of tracking dye.

Inhibition of skin collagenase

The effect of various reagents on collagenase activity was examined by using the radioactive-collagen-fibril assay. The metal-chelating agents EDTA and 1,10-phenanthroline and the thiol compounds cysteine and dithiothreitol all resulted in enzyme inhibition. Similarly, the gold salt sodium aurothiomaleate inhibited collagenase, but the thiol-blocking reagents *p*-chloromercuribenzoate and *N*-ethylmaleimide had no effect (Table 4).

Since cartilage proteoglycans were once thought to have an inhibitory effect on collagenases, we exposed the skin enzyme to relatively high concentrations of pig and human proteoglycans, but no significant inhibition was found.

Serum inhibition of skin collagenase

Whole human serum completely eliminated collagenase activity, even at dilutions approaching 200:1. Because of the possible importance of serum protein inhibitors as regulatory factors for extracellular collagenase activity *in vivo*, we have made a detailed study of the inhibitory components of serum.

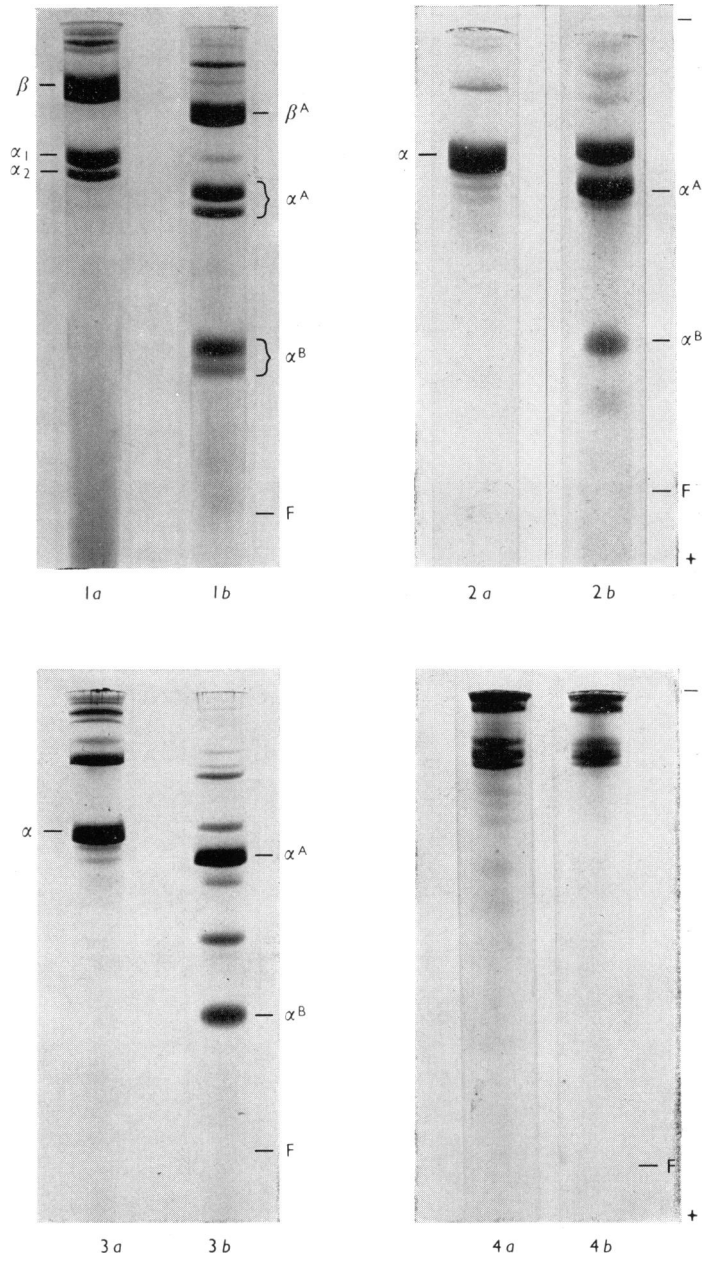
The separation of three serum anti-proteinases has been described previously (Woolley *et al.*, 1975*c*, 1976*a*), and the preparations of α_2-macroglobulin and β_1-anti-collagenase each migrated as a single band on agarose-gel electrophoresis. Addition of α_2-macroglobulin or β_1-anti-collagenase to skin collagenase resulted in almost total inhibition, the degree of inhibition being proportional to the

Table 3. *Effect of human skin collagenase on human insoluble skin collagen*

Reaction mixtures, described in the Experimental section, were incubated for 24h at 37°C and the amount of hydroxyproline released into solution and that remaining in the residues was measured. The mean and range of values from triplicate incubations are shown. The purified collagenase used in this experiment had a specific activity of 22μg of collagen degraded/min per mg of enzyme protein as judged by the collagen fibril assay.

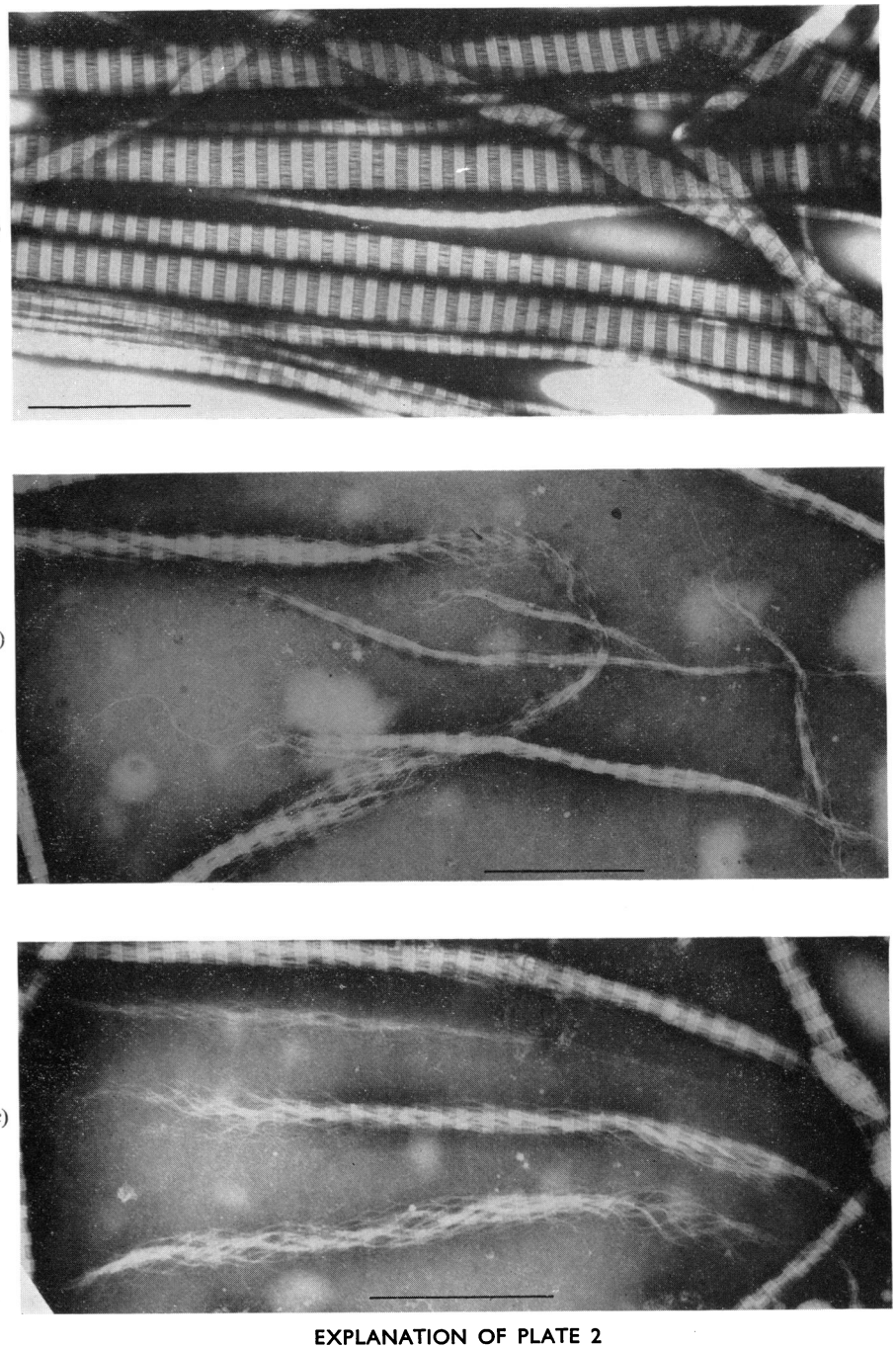
Collagen	Incubation	Hydroxyproline released (μg/24h)	Substrate degraded (%)
Insoluble skin collagen	Collagenase (150μg/ml), pH7.6	33±5	38
	Buffer, pH7.6	1±1	1
	Trypsin (75μg/ml), pH7.6	4±2	4
Reconstituted collagen fibrils	Collagenase (150μg/ml), pH7.6	650*	—

* The amount of collagen solubilized from reconstituted fibrils was extrapolated from values obtained from much shorter incubation times.



EXPLANATION OF PLATE 1

Disc gel electrophoresis of the products of skin collagenase and various collagen substrates after reaction in solution at 25°C
The control samples without enzyme (*a*) and the reaction products after exposure to collagenase (*b*) were examined in 5% polyacrylamide gels containing SDS. Each sample was equivalent to approx. 50μg of collagen. Type-I collagen (guinea-pig skin) is shown in gels 1*a* and 1*b*. Type-II collagen (chick cartilage) is shown in gels 2*a* and 2*b*. Type-III collagen (calf skin), incompletely reduced, is shown in gels 3*a* and 3*b*. Basement-membrane collagen (mouse tumour) is shown in gels 4*a* and 4*b*. Collagen types I, II and III after exposure to the purified skin collagenase yielded essentially the same characteristic products, labelled α^A and α^B. These incubations were not run simultaneously and therefore do not provide information on rates of collagen breakdown.



EXPLANATION OF PLATE 2

Electron micrographs of human insoluble collagen after exposure to purified skin collagenase at 37°C

(*a*) Insoluble collagen from human skin incubated without added enzyme; (*b*) and (*c*) insoluble collagen after incubation with purified skin enzyme for 24h at 37°C and pH7.6. All preparations were stained with 2% phosphotungstic acid, pH6.8. Bar represents 0.5 μm.

Table 4. *Effect of various agents on human skin collagenase*

Each reagent was dissolved in 50mM-Tris/HCl/0.17M-NaCl/10mM-$CaCl_2$, adjusted to pH8.0, and used in the ^{14}C-labelled-collagen-fibril assay. Collagenase (25μl) was added and the assay mixtures were incubated at 37°C for 16h. Each value represents the mean of triplicate assays after subtraction of control values and is expressed as a percentage of the fibril degradation produced by the enzyme alone. Proteoglycan concentration was calculated by measuring the uronic acid content (Hardingham & Muir, 1976). α_2-Macroglobulin and α_1-anti-trypsin concentrations were measured immunologically by single radial immunodiffusion (Mancini *et al.*, 1965), and β_1-anti-collagenase was measured by the method of Lowry *et al.* (1951).

Reagent added	Concentration in assay	Radioactivity released (c.p.m.)	Enzyme inhibition (% of control)
None	—	810	(0)
EDTA	15mM	0	100
1,10-Phenanthroline	2mM	20	98
Cysteine	2mM	40	95
Dithiothreitol	2mM	65	92
p-Mercuribenzoate	2mM	825	0
N-Ethylmaleimide	1mM	831	0
Sodium aurothiomaleate	250μg/ml	26	97
None	—	990	(0)
Proteoglycan (pig)	1.1mg/ml	998	0
Proteoglycan (human)	1.1mg/ml	900	9
None	—	1200	(0)
man serum	2% (v/v)	0	100
α_2-Macroglobulin	100μg/ml	48	96
α_1-Anti-trypsin	200μg/ml	1230	0
β_1-Anti-collagenase	100μg/ml	120	90

amount of inhibitor added. In contrast α_1-anti-
in, although inhibiting trypsin with azocasein
ubstrate, did not inhibit the collagenase even at
h[illegible] concentrations (Table 4). These findings were also confirmed by using viscometric studies, the decrease in viscosity of the collagen solution being prevented in the presence of α_2-macroglobulin or β_1-anti-collagenase, but not by α_1-anti-trypsin.

Discussion

The purification procedure described here resulted in a collagenolytic enzyme preparation with all the characteristics of a true collagenase and devoid of non-specific proteinase activity, as judged by sensitive radioassays against non-collagenous substrates. The earlier reports that more than one skin collagenase was produced by skin cultures have not been substantiated, and we have evidence for only a single enzyme being produced by skin explants. Elution profiles from gel filtration, ion-exchange chromatography and polyacrylamide-gel experiments have consistently shown the presence of only one collagenase, with mol.wt. approx. 60000. Of four human collagenases that we have examined, the skin enzyme has the largest molecular weight, values of 33000, 38000 and 54000 being obtained for the rheumatoid synovial, gastric-mucosal and granulocyte collagenases respectively (Woolley *et al.*, 1976*b*). We have no explanation for these differences in molecular size, calculated from gel-filtration experiments with and without the presence of various dissociating agents (Woolley *et al.*, 1973), but it should be noted that when comparing specific activities, differences in enzyme size should be appreciated.

Much importance has been attributed to the specific activity of collagenases as a criterion of purity (McCroskery *et al.*, 1975). Purification procedures for collagenases have usually used conventional separation techniques (e.g. Bauer *et al.*, 1970; Werb *et al.*, 1974; Woolley *et al.*, 1975*b*) and specific activities have usually been obtained from the ^{14}C-labelled collagen-fibril assay. The exceptionally high values reported by McCroskery *et al.* (1975) for their rabbit tumour collagenase may in part be explained by the inclusion of an affinity-chromatography step, which would retain only active enzyme. Our purification procedure for the human collagenase separates the enzyme on physicochemical properties, but does not have the advantage of eliminating any inactive enzyme. It is therefore apparent that to establish the purity of any enzyme preparation specific-activity data must be considered along with other criteria, such as homogeneity in polyacrylamide gels with and without SDS and the absence of non-specific proteinase activity.

The absence of non-specific proteinase activity is an important consideration when interpreting our observations on the degradation of reconstituted fibrils and insoluble highly cross-linked collagen fibres.

Reports of collagenase-mediated collagen-fibrile degradation have been questioned, in particular with respect to possible contamination of the enzyme preparations with neutral proteinase. The contention has been that collagenase is capable of only one specific cleavage across the triple-helical collagen molecule and this in itself would be insufficient to degrade reconstituted fibrils to small products or attack a highly cross-linked collagen fibre (Harris & Krane, 1974*a*,*b*,*c*; McCroskery *et al.*, 1975; Weiss, 1976).

For rheumatoid synovial collagenase a study of segment-long-spacing crystallites produced from incubations of undenatured collagen and partially purified enzyme at 25°C has shown additional attacks across the triple-helical part of the molecule at the *N*- and *C*-termini (Leibovich & Weiss, 1973). These additional cleavages may be time-dependent, occurring only after all collagen molecules have been cleaved at the usual $\frac{3}{4}:\frac{1}{4}$ locus. Our purified human skin collagenase cleaves undenatured collagen molecules at the $\frac{3}{4}:\frac{1}{4}$ locus, and at 37°C also attacks gelatinized α-chains initially at the same position. With prolonged incubations the α^A and α^B products from gelatin can be degraded further to smaller polypeptides. This action occurs even though the enzyme has no non-specific proteinase activity, as judged by sensitive radiolabelling assays, and no demonstrable activity against the synthetic oligopeptide Pz-Pro-Leu-Gly-Pro-D-Arg-OH. These findings could be explained by the presence of a contaminating gelatin-specific proteinase in our final preparation. However, such an enzyme would have to have the characteristic $\frac{3}{4}:\frac{1}{4}$ specificity of collagenase and molecular size and charge properties sufficiently similar to those of the skin collagenase to preclude its separation from it during the purification procedure. Although a gelatin-specific proteinase has been described in granulocytes (Sopata & Dancewicz, 1974) the relationship of such an enzyme to skin collagenase has not been explored. In our view the weak gelatinase activity, demonstrated after prolonged periods of incubation, is a property of the human skin collagenase itself. Just as soluble type-II cartilage collagen is six times as resistant to collagenase attack as type-I collagen (Woolley *et al.*, 1975*a*), so may other sites along gelatinized α-chains be less susceptible to enzymic cleavage.

Another point worth consideration with respect to the idea that collagenases effect only a single cleavage along the collagen molecule is that current data are based on 'hybrid' experiments using either rabbit tumour or tadpole enzymes to cleave chick, rat or human collagens (Gross *et al.*, 1974; Miller *et al.*, 1976). Our studies on degradation of insoluble collagen with either the human skin collagenase or the rheumatoid synovial enzyme (Woolley *et al.*, 1975*b*) have always used insoluble collagen fibres derived from human tissues. The possibility of subtle differences in enzyme action relative to the species of origin of collagen and enzyme is worth consideration.

In summary, we believe that although the primary action of human collagenase is at the $\frac{3}{4}:\frac{1}{4}$ locus, at physiological temperatures the resulting denatured α-chain fragments can be further degraded by the collagenase to smaller products, albeit at a much lower rate (Woolley *et al.*, 1975*b*). This is not to say that, *in vivo*, collagen degradation is accomplished only by collagenase; clearly the presence of a neutral proteinase (e.g. Harris & Krane, 1972) or a gelatin-specific proteinase (Sopata & Dancewicz, 1974) would supplement collagenase action and result in a much higher rate of fibre degradation. The 20-fold difference in the rates of collagenase attack on reconstituted fibrils and insoluble collagen fibres reported here confirms that the degree of cross-linking is very important in determining rates of degradation (Harris & Farrell, 1972).

There is now substantial evidence, with respect to collagen types I, II and III, that human collagenases do not exhibit specificity for type of collagen, although differences do exist with respect to rates of attack (McCroskery *et al.*, 1975; Miller *et al.*, 1976). The observation reported here that a purified human collagenase does not degrade basement-membrane collagen derived from mouse tumour is therefore of particular note. Since studies of the molecular structure of basement-membrane (type-IV) collagen are incomplete, and as little is known of possible species differences, the whole question of the susceptibility of human basement-membrane collagen to purified human collagenase remains to be resolved.

Because of our interest in the regulation of extracellular collagenase activity *in vivo* we attempted to identify the natural inhibitors of skin collagenase in human serum. This resulted in confirmation of the high-molecular-weight protein α_2-macroglobulin as a potent enzyme inhibitor, and also in the identification and subsequent characterization of a smaller β_1-serum protein (mol.wt. 40000), which has been shown to be a specific inhibitor of human collagenases and has been designated β_1-anti-collagenase (Woolley *et al.*, 1976*a*). Although this small inhibitor constitutes less than 5% of the collagenase-inhibiting capacity of whole serum, its greater ability to diffuse through capillary walls and permeate tissues suggests that this protein may act as an inhibitor of collagenase in tissue locations from which α_2-macroglobulin is excluded on account of its large molecular size (Woolley & Evanson, 1977).

Previous reports that skin collagenase is inhibited by α_1-anti-trypsin (Eisen *et al.*, 1970; Bauer *et al.*, 1972*b*) have not been confirmed in the present study, and no inhibitory effect was observed when this

anti-proteinase was tested against other human collagenases (Woolley *et al.*, 1976*a*). As the molecular sizes of β_1-anti-collagenase and α_1-anti-trypsin are quite similar, the earlier reports of inhibition by the latter might be explained by contamination with the β_1-protein, especially if the separation techniques used for the isolation of α_1-anti-trypsin were based only on size differences.

Of great note at the moment is the finding of natural collagenase inhibitors which are synthesized by cells or extracted directly from tissues. The molecular weights of these inhibitory components range from 6000 and 12000 in embryonic-chick skin explants (Shinkai *et al.*, 1977) and 11000 for that derived from cartilage or aorta (Kuettner *et al.*, 1976) to approx. 30000 in bone cultures (Sellers *et al.*, 1977) and 40000–45000 in tumour extracts and fibroblast cultures (McCroskery *et al.*, 1975; Bauer *et al.*, 1975). Whether these inhibitors are chemically related, e.g. as polymers, or are individually specific for collagenases, remains to be examined. Their relationship to the highly specific serum β_1-anti-collagenase has also yet to be defined.

Note Added in Proof (Received 3 November 1977)

Since this paper was prepared, an important report on precursor and active forms of skin collagenase from both fibroblast and organ cultures has appeared (Stricklin *et al.*, 1977). These authors report two forms of skin procollagenase with mol.wts. 60000 and 55000, which could be trypsin-activated to produce enzyme species of mol.wts. 50000 and 45000 respectively. Alternatively, the procollagenase forms could be autoactivated by freezing and thawing without detectable change in molecular weight. Stricklin and his co-workers concluded that the collagenase species obtained from both fibroblast and organ-culture media had identical physicochemical properties.

Our studies, reported here, have revealed only one active collagenase from organ cultures having mol.wt. 60000. We have not looked for proenzymes or latent precursors in our system. The failure of Stricklin *et al.* (1977) to elute collagenase activity or to observe stained protein bands after electrophoresis of their purified skin enzyme in basic polyacrylamide gels (Davis, 1964) is at variance, not only with the data reported here, but also with that of the same authors, who had previously described their purified skin collagenase as showing 'slow-moving' and 'fast-moving' bands in similar gels (Bauer *et al.*, 1970). The reason for these differences remains to be resolved. Our data agree with the observation that skin collagenase is a basic protein (Stricklin *et al.*, 1977), but ion-exchange chromatography and isoelectric focusing studies have shown it to be less basic than the rheumatoid synovial or gastric-mucosal collagenases (Woolley *et al.*, 1973, and unpublished work).

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