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# Purification and Characterization of a Collagenase Extracted from Rabbit Tumours

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1. A collagenase was purified from homogenates of $V_2$ ascites-cell carcinoma growing in rabbit muscle. $(NH_4)_2SO_4$ precipitation, ion-exchange and gel-filtration chromatography, and affinity chromatography (by using the CB7 CNBr cleavage fragment of α1(I) collagen linked to agarose) gave a 268000-fold purification and a sevenfold increase in total enzyme units recovered. The specific activity, defined as μmol of collagen in solution cleaved/h per mg of enzyme at 35°C, was 1.74. 2. The collagenase had a broad pH optimum from pH 7.0 to 9.5, and a mol.wt. of between 33000 and 35000. It was inhibited by dithiothreitol, L-cysteine, D-penicillamine, EDTA and 1,10-phenanthroline, and by both rabbit and human serum. 3. Removal of cations by a chelating resin (Chelex 100) produced an inactive enzyme that could be reactivated by the addition of $Ca^{2+}$ ions at concentrations as low as 1 μM. Other bivalent cations were not effective. 4. The purified collagenase cleaved peptides α2 and α1-CB7 (denatured polypeptides of collagen) at 37°C at one site only. $[\alpha1(I)]_2\alpha2$ and $[\alpha1(III)]_3$ collagens in solution were cleaved at the same site approximately five times more rapidly than $[\alpha1(II)]_3$. 5. An inhibitor of the enzyme in the tumour extracts, which was dissociable from the enzyme at the $(NH_4)_2SO_4$ precipitation step of purification, had a mol.wt. of between 40000 and 50000 but was distinct from the $\alpha_1$ trypsin inhibitor. 6. Studies with zonal density-gradient centrifugation suggested that the enzyme was bound to fibrillar substrate (collagen) extracellularly, but that it was not associated with enzymes originating in cell mitochondria, microsomal preparations or lysosomes.

A specific collagenase has been found in homogenates of tumours resulting from the implantation of $V_2$ ascites-cell carcinoma in the muscle of rabbits (Harris *et al.*, 1972). Unlike collagenases isolated from the medium of primary tissue cultures (for a review see Harris & Krane, 1974*a*,*b*,*c*), this rabbit tumour collagenase was not produced by primary cultures of the tumours. The tumour-associated enzyme was found to sediment at low *g* forces with the debris fraction containing 5′-nucleotidase and hydroxyproline-containing material, which suggests that it was bound either to cellular membranes or to collagen fibrils (Harris *et al.*, 1972).

Purified preparations of this collagenase (McCroskery *et al.*, 1973) degraded native collagen in helical conformation at 37°C into two characteristic fragments at a rate greater than it cleaved denatured collagen (i.e. gelatin). These results suggested the following hypothetical role for collagenases, namely that they cleave through the collagen triple helix at one locus, producing a 75% fragment ($TC^A$) and a 25% piece ($TC^B$). It is likely that, once cleaved *in vivo*, the collagen fragments are thermally denatured to gelatin polypeptides, and are then catabolized further by neutral endopeptidases or intracellular lysosomal enzymes.

In the present paper we describe the purification of the rabbit tumour collagenase, document some of its characteristics as a protein and enzyme, present data from studies of its localization in tumours, and show the specificity of its action on various collagen and gelatin substrates.

## Materials and Methods

### *Reagents*

$(NH_4)_2SO_4$ and sucrose were obtained from Schwarz–Mann (Orangeburg, N.Y., U.S.A.). Reagent-grade Tris, EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], glycylisoleucine, phenylmethanesulphonyl fluoride, chymotrypsinogen and 2-mercaptoethanol were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Trypsin (treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one), soya-bean trypsin inhibitor and bacterial collagenase were obtained from Worthington Biochemicals (Freehold, N.J., U.S.A.). [$^{14}$C]-Glycine and $^{14}$C-labelled mixed amino acids were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.). Minimum essential medium (MEM) was purchased from Grand Island Biological Corp. (Grand Island, N.Y., U.S.A.).

Acrylamide and methylenebisacrylamide were obtained from Canalco (Rockville, Md., U.S.A.), and recrystallized from chloroform and acetone respectively. Sodium dodecyl sulphate was purchased from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). Ampholytes (pH7–10 and pH5–8) were obtained from LKB Instruments (Rockville, Md., U.S.A.).

*Preparation and purification of the collagenase*

Undiluted ascitic fluid (3ml; approx. $30\times10^6$ tumour cells) from rabbits bearing the ascitic form of $V_2$ carcinoma, derived from the Shope papilloma virus, was injected into thigh muscles of New Zealand White rabbits (Harris *et al.*, 1972). These rabbits were provided by Dr. Sumner Wood and his colleagues at the Merck Institute for Research, Rahway, N.J., U.S.A. After 3 weeks animals with large palpable tumours were killed, and the tumour tissue was dissected away from muscle and homogenized at 4°C in 0.1M-Tris–HCl (pH7.6)–0.2M-NaCl–5mM-$CaCl_2$–0.02% sodium azide (referred to below as Tris–NaCl–$CaCl_2$ buffer) in a Polytron PT-35/2 (Brinkmann) homogenizer at high speed for two 30s bursts. The homogenate was quickly frozen and thawed, and the resulting suspension was centrifuged at 20000*g* for 30min. The supernatant was pooled with that from a second extract after additional homogenization, and was used as a source of crude collagenolytic activity. Additional enzyme could be solubilized by extraction with 3M-NaSCN (Abe & Nagai, 1973) or by separation with butan-1-ol (Morton, 1953). In the latter method the pellet was homogenized again in 0.43vol. of butan-1-ol at 4°C. After aqueous and butan-1-ol layers had separated, the aqueous layer was dialysed against Tris–NaCl–$CaCl_2$–$NaN_3$ buffer (500vol.) and concentrated in Aquacide II (Calbiochem, Spring Valley, N.Y., U.S.A.) before being assayed for collagenase.

Enzymic activity was precipitated between 20% and 50% saturation with $(NH_4)_2SO_4$ (100% saturation = 62.6g/100ml at 4°C), and the pellet after centrifugation was resuspended in 0.05M-Tris–HCl (pH7.6)–0.05M-NaCl–5mM-$CaCl_2$. After dialysis and clarification by centrifugation, the enzymic activity was applied to a column (2.5cm×30.0cm) of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.) equilibrated with the same buffer at 20°C. The activity was not retarded under these conditions. Fractions containing enzyme were reprecipitated in 60% (w/v) $(NH_4)_2SO_4$ at 4°C, and were rechromatographed on DEAE-Sephadex A-50 by using a linear gradient (200ml) from 0.035M-Tris–HCl (pH7.6)–5mM-$CaCl_2$ to 0.05M-NaCl–0.05M-Tris–HCl (pH8.6)–5mM-$CaCl_2$.

At this stage, the fractions containing collagenase were combined, placed in dialysis membranes [(Visking Corp., Chicago, Ill., U.S.A.), prepared by boiling in 1% (w/v) $NaHCO_3$] and concentrated by using Aquacide II at 4°C. The concentrate was fractionated on a column of Bio-Gel A-1.5m (Bio-Rad).

Further purification was performed with collagen–Sepharose (Bauer *et al.*, 1971), or chicken skin peptide α1-CB7 (a gift from Dr. Andrew Kang, Memphis, Tenn., U.S.A.) linked to Affi-Gel-10 (Bio-Rad) as an affinity matrix. Affi-Gel-10 is agarose with activated carboxyl-*N*-hydrosuccinimide ester linked by an ether coupling (Steers *et al.*, 1971). Linkage of peptide α1-CB7 to the Affigel-10 was performed at 4°C in 25ml of 0.1M-sodium phosphate (pH7.0) by using a 1:200 (w/w) ratio of peptide α1-CB7 to Affi-Gel-10. After the mixture had been shaken for 12h, the reaction was stopped with 1M-ethanolamine–HCl (pH8.0). The sample was applied in 0.05M-Tris–HCl (pH7.6)–5mM-$CaCl_2$ at 12°C, washed with 20-fold (v/v) amount of application buffer and the enzyme activity was collected in a batch elution with 0.1M-Tris–HCl (pH7.6)–0.75M-NaCl–0.01M-$CaCl_2$.

*Assays for collagenase activity*

Collagenolytic activity was estimated as a routine either by the use of reconstituted $^{14}C$-labelled collagen fibrils at 37°C (Harris *et al.*, 1969) or by viscometry at 35°C. At this temperature, in solution, and at neutral pH values, native mammalian collagen undergoes insignificant denaturation over the time-period (2h) needed for assay, yet the two fragments of each molecule produced by mammalian collagenases are thermally denatured. In denatured form (as random coils) the fragments contribute little to the viscosity of a solution; whereas collagen molecules have an intrinsic viscosity of >10dl/g, gelatin chains have an intrinsic viscosity of <0.4dl/g (Lewis & Piez, 1964). Viscometry was carried out in Cannon semi-micro viscometers with a flow time (water, 35°C) of approx. 1min. The temperature was kept constant at 35°C±0.1°C by using Cannon viscometric baths and Haake thermoregulators. Reaction mixtures (1ml) contained 400μg of acid-soluble guinea-pig skin collagen (1.3nmol) in the Tris–NaCl–$CaCl_2$ buffer. L-Arginine was added to a final concentration of 0.05M to prevent spontaneous collagen-fibril formation in the viscometer (Gross & Kirk, 1958; McCroskery & Harris, 1973). After conversion of flow times in seconds into percentage of initial specific viscosity ($\eta_{sp.}$), the results were plotted against time, and from such a graph the initial rate of substrate cleavage could be calculated.

Collagen degradation was also monitored by electrophoresis of reaction products (20μg) in polyacrylamide gels, prepared from 10% (w/v) acrylamide+0.135% methylenebisacrylamide, run in 0.1% sodium dodecyl sulphate in 0.05M-sodium phosphate buffer, pH7.0, generally as described by

Weber & Osborn (1969). Samples for electrophoresis were prepared by the addition of sodium dodecyl sulphate to a final concentration of 1% and 2-mercaptoethanol to 5% (v/v) before heating at 45°C for 1h. Pyronin Y was used as a tracking dye during electrophoresis at a current of 3mA/tube (internal diameter 4mm).

After electrophoresis, gels were fixed in 15% (w/v) trichloroacetic acid, stained at 18°C in 0.1% Coomassie Blue R-250 in methanol–acetic acid–water (5:1:4, by vol.) for 2h, and then de-stained at 28°C in aq. 7.5% acetic acid and 5% methanol.

Certain gels to which radioactive collagen had been applied were sliced with a razor blade, and the fragments were placed in 10ml portions of Bray's (1960) scintillant solution containing 0.5ml of NCS (Nuclear–Chicago, Chicago, Ill., U.S.A.) with 0.1ml of 1M-$NH_3$ and counted for radioactivity in a liquid-scintillation counter (Packard Instruments, Downers Grove, Ill., U.S.A.).

*Electrophoresis of non-collagen proteins*

Polyacrylamide-gel electrophoresis, in the presence of sodium dodecyl sulphate, of samples from various steps of enzyme purification were performed as described by Weber & Osborn (1969). Disc-gel electrophoresis by the method of Davis (1964) was also used.

*Isoelectric focusing in polyacrylamide gels*

Gels for isoelectric focusing were prepared in glass tubes (0.5cm×12cm) by using 2% (w/v) pH7–10 ampholytes and 0.4% pH5–8 ampholytes (LKB Instruments) with 7.5% (w/v) acrylamide, 0.2% methylenebisacrylamide. $(NH_4)_2S_2O_8$ (0.5%) was used for polymerization. The upper (cathode) buffer was 0.4% ethylenediamine and the lower (anode) buffer was 0.2% phosphoric acid. Gels were pre-run for 30min at 4°C (1mA/tube). The sample to be analysed was introduced in 15% (w/v) sucrose under a 0.2ml layer of 1% pH7–10 ampholytes in 5% sucrose and run for 4h, with a maximum current of 1mA/tube. During each run the voltage was decreased from 400 to 100V. For the detection of protein, the gels were placed in 10% (w/v) trichloroacetic acid–3% sulphosalicylic acid at 60°C for 45min, stained with 0.1% Coomassie Blue in 50% methanol, and de-stained in 20% ethanol–aq. 7.5% acetic acid. A companion gel, containing the same sample, was sliced into 1mm sections (with a Bio-Rad gel slicer) and incubated with 0.2ml of 0.1M-Tris–HCl (pH7.6)–0.5M-NaCl–0.01M-$CaCl_2$ for 3h, and was assayed for collagenase activity with collagen in solution at 35°C as substrate. Reaction products were detected by gel electrophoresis by the system described by Nagai *et al.* (1964).

*Collagen and gelatin substrates*

$^{14}$C-labelled collagen extracted by NaCl or acetic acid from guinea-pig skin was purified as described (Gross, 1958; Glimcher *et al.*, 1964; Harris *et al.*, 1969). This material was assumed to have a molecular composition primarily of $[\alpha1(I)]_2\alpha2$. CNBr-cleaved peptides ($\alpha$1-CB7 and $\alpha$1-CB8) of chicken skin $\alpha$1(I) chains were kindly supplied by Dr. Andrew Kang (Memphis, Tenn., U.S.A.); $\alpha$2 chains from rat skin collagen were a gift from Jerome Gross (Boston, Mass., U.S.A.); pepsin-extracted $[\alpha1(II)]_3$ collagen from human and bovine articular cartilage and $[\alpha1(III)]_3$ collagen (Chung & Miller, 1974) from human leiomyomata were kindly given by Dr. Edward Miller (Birmingham, Ala., U.S.A.) [for references to collagen chain and CNBr-cleaved peptide nomenclature see Harris & Krane (1974*a*,*b*,*c*) and Traub & Piez (1971)].

*Immunodiffusion*

Double immunodiffusion was performed at room temperature (20°C) on agar-coated slides, by using gels of 1% agarose in potassium–sodium phosphate buffer (20mM, pH6.0) in 0.15M-NaCl (Ouchterlony, 1962). Guinea-pig antiserum against rabbit $\alpha_1$ trypsin inhibitor was a generous gift from Dr. F. Kueppers (Rochester, Minn., U.S.A.). Immunoglobulins from antiserum were purified by precipitation with 45%-satd. $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose (Whatman DE-52; Whatman Biochemical, Maidstone, Kent, U.K.) equilibrated with 0.03M-sodium phosphate buffer (pH8.3). Antiserum to rabbit immunoglobulin G was purchased from Difco Laboratories (Detroit, Mich., U.S.A.).

*$Ca^{2+}$-free collagenase*

Collagenase free of $Ca^{2+}$ was prepared by dialysis against 0.1M-Tris–HCl (pH7.6)–0.2M-NaCl (twice recrystallized) followed by passage over a column (0.9cm×5cm) of Chelex-100 ($Na^+$ form; Bio-Rad) equilibrated with 0.05M-Tris–HCl (pH7.6). All solutions were made with double-distilled water that had been treated with Chelex. Plastic vessels, washed with $HNO_3$ (1%)–HF (0.5%) and rinsed with Chelex-treated water, were used throughout. Collagen used for the assay of the $Ca^{2+}$-free collagenase was dialysed three times against 500vol. of 0.1M-Tris–HCl (pH7.6)–0.2M-NaCl (recrystallized).

*pH-dependence determinations*

Buffer solutions were prepared by the addition of acetic acid (1.0M) to a solution of 0.2M-Tris–0.4M-NaCl–0.01M-$CaCl_2$ to give the desired pH, then by

adding water to give a final concentration of 0.1M-Tris–0.2M-NaCl–5mM-$CaCl_2$. The pH of the final solutions was measured both at room temperature and at 37°C, the assay temperature. Collagenase solutions were dialysed against 25mM-Tris-HCl (pH7.6) before assay, and added to 0.1% salt-soluble collagen in a volume not more than one-quarter that of the total. The collagenase used had been purified through the peptide α1-CB7–agarose affinity-chromatography step. The enzyme/substrate ratio (w/w) was approx. 1:25. After incubation at 37°C the reaction products were run on polyacrylamide gels (Nagai *et al.*, 1964) and the percentage of collagen molecules cleaved at each pH was estimated visually.

*Separation of a collagenase inhibitor from tumour homogenates*

After the collagenolytic activity was precipitated from crude homogenates in a 20–50%-satd. $(NH_4)_2SO_4$ fractionation, material inhibitory to collagenase activity was precipitated in a 75–90%-satd. $(NH_4)_2SO_4$ fraction. This pellet was dissolved in, and dialysed against, Tris–NaCl–$CaCl_2$ buffer, and insoluble material was removed by centrifugation. The inhibitory fraction then was chromatographed on a column (55cm×2.5cm) of Bio-Gel A-1.5m. The fractions were assayed for inhibitory activity against collagenase by the $^{14}$C-labelled-collagen-fibril assay described above. A portion (100μl) of each fraction to be tested for inhibition, 50μl of tumour collagenase (44 units/mg; see Table 1 for definition of 1 unit) and 50μl of 0.1M-Tris–HCl (pH7.6)–0.2M-NaCl–5mM-$CaCl_2$–0.02% $NaN_3$ were incubated at 37°C with 100μl of reconstituted $^{14}$C-labelled collagen fibrils. For assay of anti-trypsin activity, $^{14}$C-labelled gelatin was used as substrate for 1.0μg of trypsin and 100μl of each fraction to be tested by using the gelatinase assay described by Harris & Krane (1972).

*Miscellaneous*

Protein determinations were made by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Collagen concentrations were measured by initial dry weight and confirmed by hydroxyproline analyses (Prockop & Udenfriend, 1960). Amino acid analysis of various proteins was kinldy performed by Dr. Marvin Tanzer (Hartford, Conn., U.S.A.). Assays for neutral proteinase activity with $^{14}$C-labelled gelatin as substrate were performed by the method of Harris & Krane (1972). Haematoxylin and Eosin stains and Tri-chrome connective-tissue stains of tumour slices fixed in 10% formalin, were provided by the Department of Pathology Dartmouth/Hitchcock Medical Center.

*Study of localization of collagenase in tumour homogenates*

*Preparation of material for sucrose-gradient centrifugation.* Tumours removed from rabbit muscle as described above were collected in 0.15M-NaCl–0.01M-Tris–HCl (pH7.6) at 4°C. Cells were removed from stroma by three to five strokes in a Teflon–glass homogenizer [clearance 0.28mm (0.011in)], filtered through gauze pads, washed twice, resuspended in 1mM-$NaHCO_3$ (pH8.0)–5mM-$CaCl_2$ (20ml/g of cells) and stirred vigorously at 4°C for 30min. At this point less than 10% of cells were estimated to be broken (estimated from Haematoxylin and Eosin stains of the cell suspension); accordingly the suspension was treated with 10–20 strokes with a Dounce homogenizer, after which 90–95% of cells were broken. This suspension of fragmented cells was diluted with an equal volume of 1.0M-sucrose. This 'original homogenate' was centrifuged at 165*g* at 4°C for 4min. The supernatant was centrifuged at 9200*g* for 20min, and the pellet was resuspended in 0.25M-sucrose–0.01M-Tris–HCl (pH7.4)–1mM-$CaCl_2$ and applied to sucrose gradients.

*Zonal density-gradient centrifugation.* Sucrose solutions were prepared by dilution of a stock sucrose solution [66% (w/w) in 0.01M-Tris–HCl (pH7.6)–5mM-$CaCl_2$]. The following solutions (total vol. 1400ml) at 4°C were pumped into a chilled Sorvall SZ-14 reorienting density-gradient zonal rotor: buffer overlay, sample and equal quantities of 30, 40, 47, 55 and 65% sucrose. Centrifugation at 4°C was at 2665*g* for 1h or 9800*g* for 45min. After each run, the most concentrated sucrose was collected as one fraction, and the remainder of the gradient was collected in 25ml fractions that were diluted with 0.1M-Tris–HCl (pH7.6)–5mM-$CaCl_2$ and centrifuged at 27000*g* for 15min. The concentrated material forming a pellet was resuspended in a small volume of 0.1M-Tris–HCl (pH7.6)–5mM-$CaCl_2$ and assayed for protein and several or all of the following enzymes: collagenase ($^{14}$C-labelled collagen-fibril substrate), succinate dehydrogenase (Pennington, 1961), acid phosphatase (Lowry *et al.*, 1954), $Na^+$+$K^+$-activated adenosine triphosphatase (Bonting *et al.*, 1963), 5′-nucleotidase (Heppel & Hilmoe, 1951) and glucose 6-phosphatase (Nordlie & Arion, 1964). $P_i$ was assayed by the method of Fiske & SubbaRow (1925).

## Results

*Morphological description of the tumour tissue*

Some 3 weeks after implantation in thigh muscles, the tumour appeared salmon-pink and had a spongy consistency, in contrast with the surrounding pale rubbery muscle. Foci of necrosis contained dark thin fluid. Grossly, the tumours frequently invaded bone

Table 1. *Purification of rabbit tumour collagenase*

The enzyme activity was assayed with $^{14}C$-labelled collagen fibrils as substrate (see the Materials and Methods section). One unit of collagenase activity solubilized 1 mg of collagen fibrils/h at 37°C.

| | Protein | | Collagenase (units) | Specific activity (units/mg of protein) | Relative purification (fold) | Fractional recovery |
|---|---|---|---|---|---|---|
| | (mg, total) | (mg/ml) | | | | |
| 1. Combined supernatants from homogenates | 31869 | 23.6 | 26.4 | 0.0008 | 1 | 1.0 |
| 2. 20–50% satn. with $(NH_4)_2SO_4$ | 5100 | 20.4 | 262.7 | 0.05 | 65 | 10.0 |
| 3. DEAE-Sephadex A-50 | 1062 | 11.8 | 452.9 | 0.43 | 533 | 17.2 |
| 4. Bio-Gel A-1.5m | 7.14 | 0.07 | 317.1 | 44.4 | 55500 | 12.0 |
| 5. Peptide α1-CB7–agarose | 0.83 | 0.02 | 178.5 | 215.1 | 268780 | 6.7 |

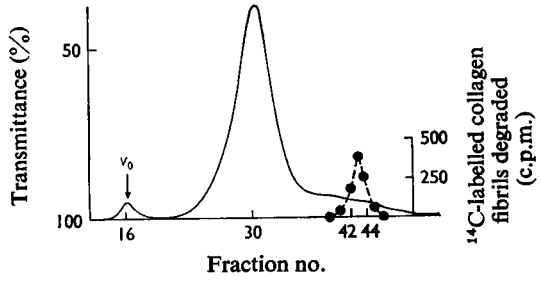



Fig. 1. *Gel chromatography of rabbit tumour collagenase on Bio-Gel A-1.5m*

The sample was first fractionated by $(NH_4)_2SO_4$ and passed through DEAE-Sephadex A-50 as described in the text. The column of Bio-Gel A-1.5m (1.5cm×55cm) was equilibrated in 0.1M-Tris–HCl (pH7.6)–0.5M-NaCl–0.01M-$CaCl_2$ with 0.2% $NaN_3$ as preservative. The column was run at 4°C; 2ml fractions were collected. $V_0$, void volume. Collagenase assays were performed on fractions 16–50; – – ●, collagenolytic activity; ——, transmittance (%) at 280nm.

and skin. In general, the separation of tumour from surrounding muscle was done easily with a spoon-spatula and dissecting scissors. Histologically, at light-microscopic magnifications, multiple bands of collagen were seen to streak through tumour cells, becoming more dense near the invasion front. There was a minimal lymphocyte response to the tumour, and no polymorphonuclear leucocytes were seen in the intact areas of tumour or in necrotic areas.

*Purification of collagenase from rabbit tumour homogenates*

Collagenolytic activity was detected in the necrotic fluid in the centre of excised tumours and in crude homogenates of the tumour mass. Approx. 85% of the total collagenolytic activity found in each tumour was recovered in Tris–NaCl–$CaCl_2$-buffer extracts of homogenates of the tissues that had been rapidly frozen and thawed. The remaining 15% of the collagenolytic activity was solubilized by dialysis against 3M-NaSCN, pH6.0, or by extraction into the aqueous phase after shaking with 0.43vol. of butan-1-ol.

Material containing collagenase activity could be precipitated between 20% and 50% saturation with $(NH_4)_2SO_4$ at 4°C. In this step a 5–20-fold increase in the total collagenolytic activity recovered was observed (Table 1). This increase in activity has been attributed to the dissociation and separation of a reversible inhibitor (see below). In subsequent stages of purification, the collagenase was often concentrated from dilute solutions by precipitation at 80% saturation with $(NH_4)_2SO_4$.

The collagenase was not adsorbed on DEAE-Sephadex A-50 under the conditions described; at this stage of purification the principal contaminating protein was immunoglobulin G, which has an isoelectric point similar to the enzyme. The immunoglobulin G was identified by double immunodiffusion against specific antiserum to rabbit immunoglobulin G after amino acid analyses of the sample had revealed a composition similar to immunoglobulin G. Finer resolution by using a second column of DEAE-Sephadex A-50 in a buffer solution of lower initial ionic strength effected additional purification of collagenase but at the expense of a 50–60% loss of activity.

Passage of the partially purified enzyme through a column of Bio-Gel A-1.5m yielded collagenase essentially free of immunoglobulin G and contaminating proteinases capable of degrading denatured collagen (Fig. 1). After this stage of purification, assays of enzyme activity could be performed at 35–37°C and evidence for only one cleavage site of the collagen molecule was found.

The enzyme could be purified further by affinity chromatography on collagen or α1-CB7 peptide linked to agarose. It was noted consistently that, although there was a higher recovery of the collagenase from collagen–Sepharose than from α1-CB7–

Table 2. *Specific activity of collagenase preparations at 35°C and pH7.6 with soluble collagen substrate*

| Sample | Enzyme: Protein (μg/ml) | Enzyme: Total (μg) | Collagen cleaved (μmol/h per mg of enzyme) |
|---|---|---|---|
| 1. DEAE-Sephadex | 11800 | 33028 | 0.00297 |
| 2. Bio-Gel A-1.5m | 64.5 | 354 | 0.277 |
| 3. Peptide α1-CB7–agarose (low *I*) | 16.5 | 331 | 0.186 |
| Peptide α1-CB7–agarose (high *I*) | 2.8 | 23.7 | 1.74 |

agarose, the total purifiction achieved was less in the former than the latter, indicating that other proteins had affinity for collagen under these conditions; one-step purifications with affinity matrices could therefore not be complete. Peptide α1-CB7 was chosen as the preferred ligand, since the binding of non-collagenase protein was less and the amount of hydrolysis of peptide α1-CB7 was insignificant at 12°C when proteinase-free samples were applied. Neither the dipeptide glycylisoleucine (0.01 M in the starting buffer) nor the chelating agent EGTA was able to remove the collagenase from the affinity matrices.

Table 1 shows that the data for relative purification are artificially inflated by the large net increase in recovery of activity (e.g. up to 17 times the original activity after the DEAE-Sephadex A-50 purification step). Disregarding the increase in total collagenase activity that occurred through the purification, a phenomenon related to separation of an inhibitor from collagenase (see below), the final relative purification was 40000, not 270000.

Plate 1 shows polyacrylamide gels of protein at various steps of purification. Not until the step using Bio-Gel A-1.5m could a discrete band consistent with collagenase be seen. Traces of contaminating protein were present (gels *e*) after the affinity-chromatography step. The apparent mol.wt. of the collagenase (gels *e* and *f*) was between 33000 and 35000. This was consistent with other gels (not shown) in which ovalbumin (mol.wt. approx. 43000) and cytochrome *c* (mol.wt. approx. 13000) were also used as marker proteins on sodium dodecyl sulphate–polyacrylamide gels.

A sample of enzyme purified on peptide α1-CB7–agarose was isoelectrically focused on two polyacrylamide gels by using ampholytes as described in the Materials and Methods section. One gel was stained and de-stained; the other was sliced and assayed for collagenase activity. Three bands (Plate 2*a*) were present on the stained gel; the pH in this area was 6.8–7.0. After a long incubation at 35°C collagenolytic activity was demonstrable in gel slices coinciding with bands on stained gels (Plate 2*b*).

Table 3. *Comparison of the effect of inhibitors on rabbit tumour collagenase and human synovial collagenase*

The human synovial collagenase was prepared from pooled tissue culture media from the third to the sixth days of primary cultures (in the absence of serum) of rheumatoid synovial tissue (Evanson *et al.*, 1967). Assays were performed with $^{14}$C-labelled collagen fibrils as substrate, and all reactions were stopped by centrifugation when control reactions containing enzyme and substrate alone were 70–80% degraded. Rabbit serum was taken from an animal bearing a tumour. Except for serum, all inhibitors were added in solution at pH7.6 to give a final concentration of 0.010 M.

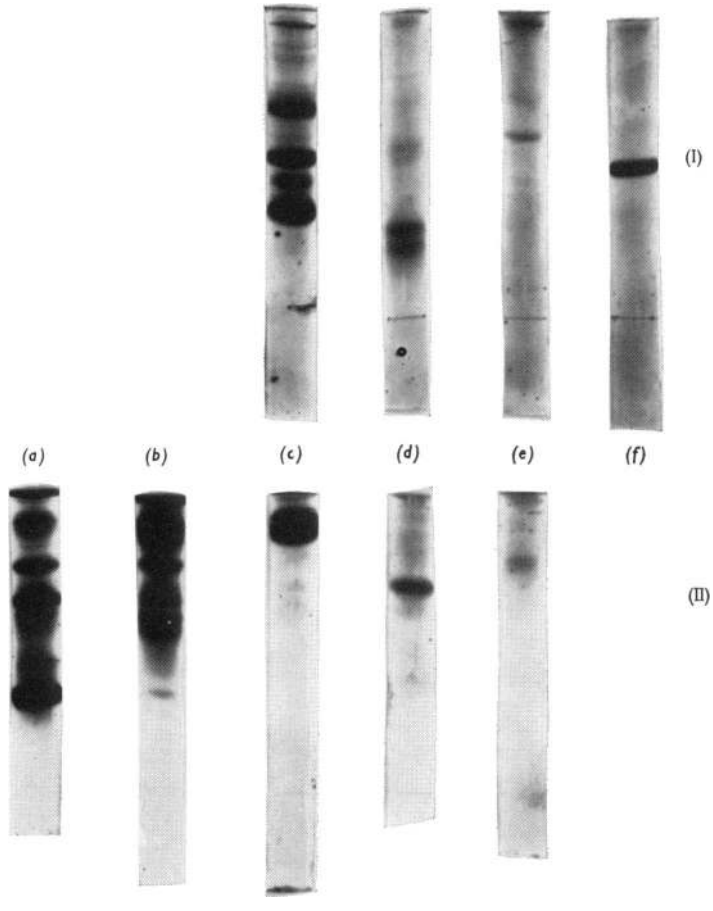
| Inhibitors | Inhibition (%): Synovial collagenase | Inhibition (%): Tumour collagenase |
|---|---|---|
| Dithiothreitol | 86 | 79 |
| L-Cysteine | 55 | 36 |
| D-Penicillamine | 73 | 73 |
| EDTA | 87 | 84 |
| L-Histidine | 5 | 28 |
| 1,10-Phenanthroline | 96 | 94 |
| Serum, rabbit (1:20) | 75 | 76 |
| Serum, human (1:20) | 61 | 70 |

*Specific activity of purified tumour collagenase*

In Table 2 the specific activity of various preparations of the collagenase are presented. Similar to the results in Table 1 determined with fibrillar collagen as substrate, the greatest increase in specific activity occurred at the gel-filtration step. In the affinity-chromatography steps, much of the enzyme was not adsorbed on the matrix at low ionic strength, but a sixfold purification was achieved in the high-ionic-strength wash. Comparative assays with identical enzyme preparations suggested that the rate of cleavage of collagen in solution at 35°C was approximately twice that of reconstituted fibrils at 37°C. It is noteworthy that partially purified collagenase has been found to be relatively unstable in solution at 4°C or frozen at −20°C. The pure enzyme, however, after purification with the use of peptide α1-CB7–agarose, has been found to retain 90% of its activity when stored as long as 1 year at −20°C.

*pH-dependence*

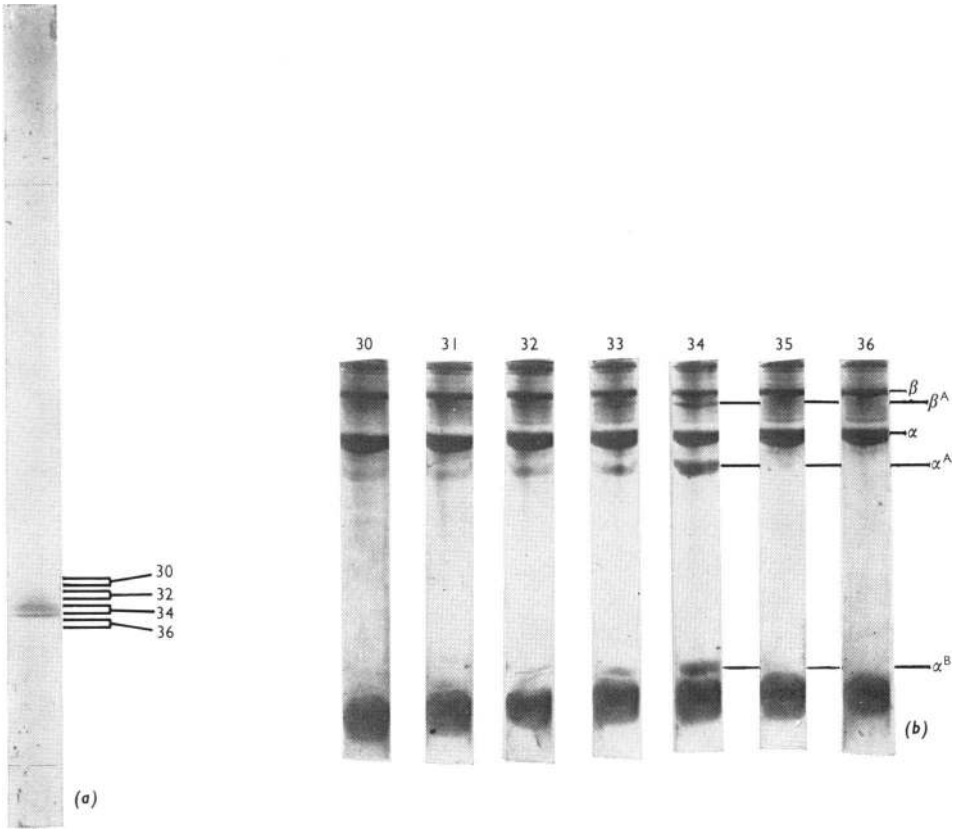
With collagen in solution as substrate, and by estimating rates of degradation visually on polyacrylamide gels, the pH maximum was found to be 8.0 in a broad range of 80% maximum activity between pH7.0 and 9.5.




## EXPLANATION OF PLATE 1

*Polyacrylamide gels of rabbit tumour from each purification step comparing charge distribution and molecular size of proteins present*

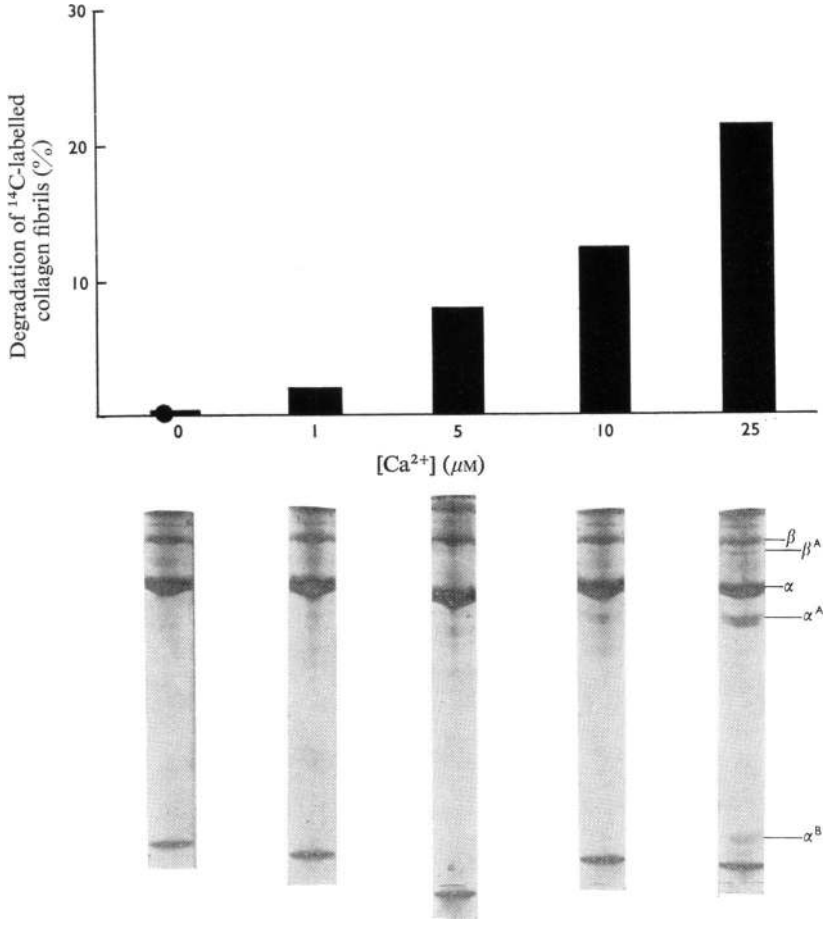
Gels in the upper row (I*c*, I*d*, I*e*, I*f*) were run in the presence of sodium dodecyl sulphate; those in the lower row (II*a*, II*b*, II*c*, II*d*, II*e*) had no sodium dodecyl sulphate present (Davis, 1964). Samples applied to each gel were as follows: II(*a*), rabbit serum; II(*b*), material precipitated by 20–50%-satd. $(NH_4)_2SO_4$; I(*c*) and II(*c*), material containing collagenase eluted in the starting buffer from DEAE-Sephadex A-50; I(*d*) and II(*d*), collagenase peak from Bio-Gel A-1.5m (see Fig. 1); I(*e*) and II(*e*), collagenase eluted from peptide α1-CB7–agarose columns in 0.1M-Tris–HCl (pH7.6)–0.75M-NaCl–5mM-$CaCl_2$; I(*f*), chymotrypsinogen (estimated mol.wt. 25700).




## EXPLANATION OF PLATE 2

*Isoelectric focusing of purified collagenase*

(*a*) is a stained gel after isoelectric-focusing electrophoresis of purified rabbit tumour collagenase; (*b*) shows corresponding activity of enzyme, eluted from slices of a similar gel, demonstrated by electrophoresing the products on polyacrylamide gels. Reaction mixtures, incubated at 35°C for 45h, contained 0.05ml of 0.4% salt-soluble guinea-pig skin collagen, 0.1ml of eluate from each gel slice and 0.15ml of 0.1M-Tris–HCl (pH7.6)–0.01M-$CaCl_2$. Gel no. 32 broke near the buffer front during transfer. The heavily staining band at the buffer front represents ampholytes.



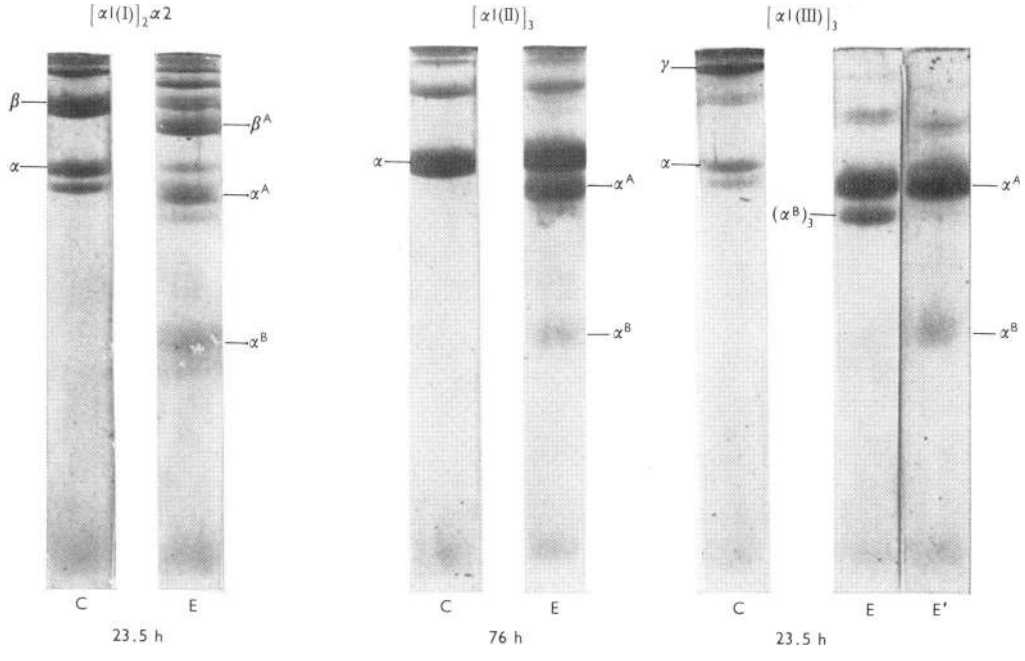

## EXPLANATION OF PLATE 3

*Restoration of collagenolytic activity to enzyme after treatment with Chelex-100 by the addition of $Ca^{2+}$*

The upper bar graph represents the percentage of collagen degraded after addition of $Ca^{2+}$ to inactive preparations of enzyme in final concentration as shown. The assay for collagenolytic activity was at 27°C and guinea-pig $^{14}$C-labelled skin collagen in solution was used as substrate. Two polyacrylamide disc gels were run on each sample reaction of products. One gel was stained as shown; the other was frozen, sliced, and radioactivity in slices was counted as described in the text. Percentage lysis was determined by determining:

$$\frac{(\frac{4}{3}\text{c.p.m.}\alpha^A)}{(\text{c.p.m.}\alpha)+(\frac{4}{3}\text{c.p.m.}\alpha^A)} \times 100$$

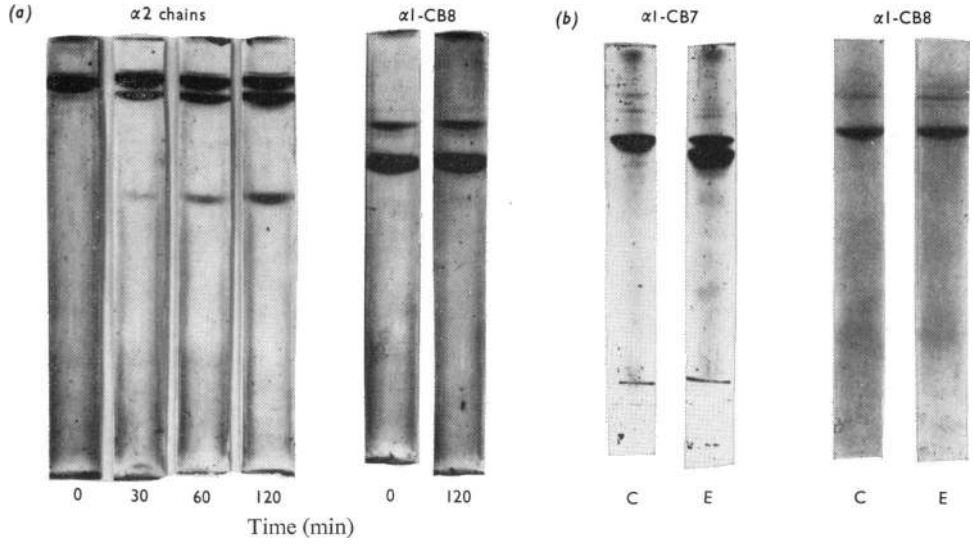
It was necessary to multiply c.p.m. appearing as $\alpha^A$ by 4/3 because of the greater mobility on gels of $\alpha^B$ (representing 25% of each cleaved molecule), which was not counted for radioactivity.




## EXPLANATION OF PLATE 4

*Reaction products on sodium dodecyl sulphate–polyacrylamide gels of rabbit tumour collagenase and three types of collagen*

C, Without enzyme; E, with enzyme. All samples were run on 5% polyacrylamide gels in the presence of sodium dodecyl sulphate. The two gel electrophoretograms of reaction products from enzyme plus [α1(III)]$_3$ are before (E) and after (E′) reduction of the reaction products in 0.1% 2-mercaptoethanol at 25°C for 1 h before electrophoresis.




EXPLANATION OF PLATE 5

*Degradation of denatured components of* $[\alpha 1(I)]_2\alpha_2$ *collagen by purified rabbit tumour collagenase at* 37°C

The substrate/enzyme ratio (w/w) for each assay was approximately 50:1, although different enzyme preparations were used for (*a*) and (*b*). Estimated mol.wts. for the substrates were: α2, 95000; α1-CB8, 23000; α1-CB7, 25000. The reaction products of α2 chains were run on 5% polyacrylamide gels, those of α1-CB8 and α1-CB7 in 10% polyacrylamide, all in sodium dodecyl sulphate. Incubation time for the α1-CB7 and α1-CB8 in (*b*) was 26h. C, Without enzyme; E, with enzyme.

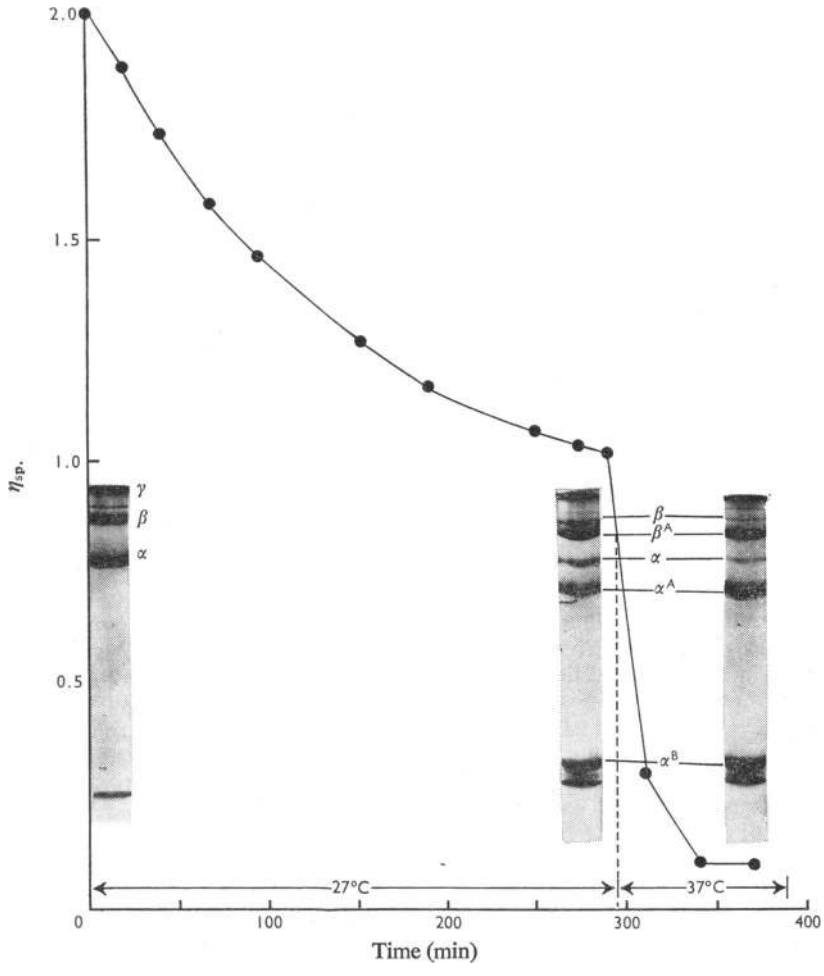



Fig. 2. *Viscosity ($\eta_{sp.}$ of cleaved collagen at 27 and 37°C*

The first gel on the left represents the composition of reaction at zero time: the middle gel shows reaction products at 27°C at 295min; the final gel shows reaction products after 360min of the reaction, 65min of which was at 37°C. The incubation mixture consisted of 800$\mu$g of acid-soluble guinea-pig skin collagen and 10$\mu$g of purified tumour collagenase free from proteinase activity (see the text). A 2 molar excess of EDTA to $Ca^{2+}$ was added to each portion before electrophoresis on gels.

*Inhibitors of collagenase activity*

Table 3 shows comparative inhibition of rabbit tumour and human synovial collagenases by chelating agents, thiol compounds and serum. The synovial enzyme was chosen for comparison because it has a similar molecular weight to that of the rabbit tumour enzyme. The similarity in the amount of inhibition of both enzymes by the same compounds was found with all inhibitors tested. 1,10-Phenanthroline consistently inhibited both enzymes more effectively than EDTA at the same molar concentration. Inhibition by 1,10-phenanthroline could not be reversed by addition of an excess of $Ca^{2+}$, yet, if a mixture of rabbit tumour collagenase in 1mM-1,10-phenanthroline were dialysed exhaustively at 4°C against 0.1M-Tris–HCl (pH7.6)–0.010M-$CaCl_2$, 90% of the original collagenolytic activity was restored.

*$Ca^{2+}$-free collagenase*

Collagenase was inactivated by passage through the chelating resin Chelex-100. These preparations were inactive against native collagen substrate in solution, yet activity was immediately restored by the

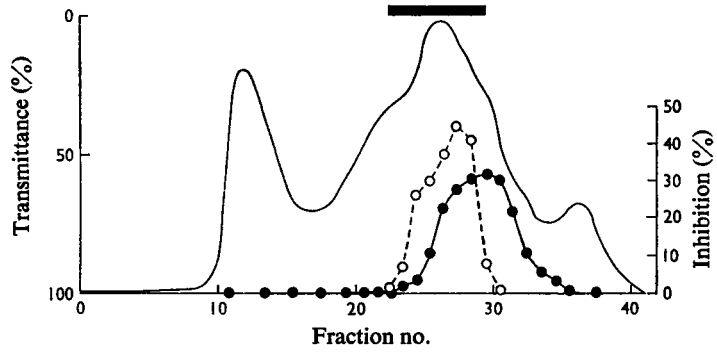



Fig. 3. *Gel-filtration chromatography of collagenase inhibitor in the 75–90%-satn.-$(NH_4)_2SO_4$ pellet from tumour homogenates*

The Bio-Gel A-1.5m column (110ml) was run in 0.1M-Tris–HCl (pH7.6)–0.5M-NaCl–0.01M-$CaCl_2$ at 4°C; 2.8ml fractions were collected. Collagenase inhibition (●) and inhibition of degradation of gelatin (○) by trypsin are plotted as percentage inhibition. The same $^{14}C$-labelled substrate was used in native form for the collagenase assay and in denatured form for the assay of gelatin degradation. Immunoreactivity with guinea-pig anti-(rabbit $\alpha_1$ trypsin inhibitor) was limited to fractions included by the horizontal bar.

addition of small amounts of $CaCl_2$. Plate 3 demonstrates the graded response of Chelex-treated enzyme to addition of increasing quantities of $Ca^{2+}$. The activity restored at 25$\mu$M-$Ca^{2+}$ represented approx. 10% of original activity. After 21h at 35°C, the $Ca^{2+}$-free enzyme could be restored to 75% of its original activity by the addition of 1mM-$CaCl_2$, and control samples of collagenase were 90% as active as original samples after incubation under the same conditions. It was concluded that the enzyme was somewhat stabilized, as well as activated, by $Ca^{2+}$.

The specificity of $Ca^{2+}$ was studied by using other cationic salts, namely $ZnCl_2$, cobalt acetate, magnesium acetate, $MnSO_4$ and $CuSO_4$. In the presence of an equimolar concentration of EGTA (1mM) (used for its specificity as a chelator of $Ca^{2+}$), none of these salts restored significant activity to the Chelex-treated enzyme.

*Activity of purified rabbit tumour collagenase against different types of intact collagens, denatured collagen chains and CNBr peptides of collagen*

As shown in Fig. 2, rabbit tumour collagenase used after the Bio-Gel A-1.5m purification step produced reaction products consistent with one cleavage through $[\alpha1(I)]_2\alpha2$ collagen at 27°C, and, after denaturation of the reaction products by warming the incubating bath to 37°C, the appearance of the reaction products did not change except for a small increment in the extent of the reaction. We have observed consistently that, if any non-specific proteinase remained associated with the collagenase, reaction products smaller than $\alpha^A$ were easily demonstrated on this type of gel.

Plate 4 shows reaction products of purified rabbit tumour collagenase incubated with collagen from guinea-pig skin $\{[\alpha1(I)]_2\alpha2\}$, human articular cartilage $\{[\alpha1(II)]_3\}$, and human leiomyoma $\{[\alpha1(III)]_3\}$. The last-mentioned collagen contains three cysteine residues/molecule, and these experiments demonstrate that thiol cross-links are in the *C*-terminal 25% of $[\alpha1(III)]_3$ collagen; after the reaction, two new bands were produced from the highly aggregated original form, both with apparent mol.wt. of about 75000. However, after reduction by 2-mercaptoethanol of the entire reaction mixture, one band consistent with $TC^A$ fragment remained and a band consistent with a mol.wt. of 25000 appeared. Our initial experiments showed no significant differences between the rate of cleavage of peptides $[\alpha1(I)]_2\alpha2$ and $[\alpha1(III)]_3$, but cartilage collagen $[(\alpha1(II)]_3$ was degraded at a rate one-fifth of that for types I and III.

Denatured collagen (i.e. gelatin) was degraded at a rate approximately one-twentieth that for helical collagen when both were cleaved by equal amounts of purified collagenase. Plate 5 shows sodium dodecyl sulphate–polyacrylamide-gel electrophoretograms of reaction products of purified rabbit tumour collagenase and $\alpha2$ chains and $\alpha1$-CB7 and $\alpha1$-CB8 peptides. $\alpha1$-CB7 peptide was chosen because it contains the cleavage site predicted from knowledge of (*a*) the alignment of peptides produced by action of CNBr and (*b*) where the cleavage of collagen by vertebrate collagenases occurs, by using electron microscopy of segment long-spacing aggregates. All these reactions took place at 37°C, and it appeared that there was no non-specific cleavage of peptides $\alpha2$ or $\alpha1$-CB7 at sites other than the primary cleavage site. The fragment bearing a new *N*-terminus produced by

Table 4. *Effect of addition of fibrillar collagen to homogenates on distribution of collagenase*

Composition of each system is detailed in the text. Tris–NaCl–$CaCl_2$ buffer 0.01 M Tris–HCl (pH 7.6)–0.15 M-NaCl–5 mM-$CaCl_2$.

| Solution for centrifugation | Original total enzyme activity in supernatant (%) | | | |
|---|---|---|---|---|
| | Succinate dehydrogenase | Acid phosphatase | 5′-Nucleotidase | Collagenase |
| (a) Tris–NaCl–$CaCl_2$ | 10.2 | 22.4 | 12.5 | 37 |
| (b) Tris–NaCl–$CaCl_2$+0.25 M-sucrose | 15.5 | 25.7 | 16.6 | 37 |
| (c) Tris–NaCl–$CaCl_2$+0.40 M-sucrose | 12.4 | 27.6 | 16.6 | 38 |
| (d) Tris–NaCl–$CaCl_2$+collagen fibrils | 10.0 | 21.9 | 12.5 | 18 |

cleavage of peptide α1-CB7 was small, and probably diffused out of the gels during de-staining; however, the small fragment from this particular reaction (Plate 5) has been isolated and the new *N*-terminal sequence at the cleavage site determined (Gross *et al.*, 1974).

*Dissociable inhibitors of collagenase found in tumour homogenates*

As noted above (Table 1), a 17-fold increase in total collagenase activity was found after the DEAE-Sephadex stage of purification compared with that present originally. In addition, 10-fold greater total enzyme activity could be recovered from a second extraction of the tumour homogenate by 0.1 M-Tris–HCl (pH 7.6)–0.2 M-NaCl–5 mM-$CaCl_2$–$NaN_3$ (0.02%) than was recovered in the first extract. We inferred from these results that inhibitory material was present in the homogenates but could be dissociated from enzyme by standard methods of salt precipitation and chromatography. Accordingly, other protein precipitated from solution in different saturations of $(NH_4)_2SO_4$ was tested for inhibitory activity against collagenase. A collagenase inhibitor was found in the 75–90%-satn.-$(NH_4)_2SO_4$ precipitate. It was eluted from a column of Bio-Gel A-1.5 m in a volume corresponding to a mol.wt. of 40000–50000. Fractions from this column were tested for capacity to inhibit the breakdown of $^{14}C$-labelled collagen fibrils by collagenase and $^{14}C$-labelled gelatin by trypsin; quantitative assessment of these two inhibitory capacities did not overlap precisely (Fig. 3). We used guinea-pig anti-(rabbit $\alpha_1$-trypsin inhibitor) to demonstrate that the two fractions containing the peak of immunoreactive material had no inhibitory activity against collagenase, although they did inhibit the action of trypsin on gelatin. Conversely, peaks of collagenase-inhibiting substances had little capacity to inhibit gelatin degradation by trypsin. Activity of the collagenase inhibitor substance was not diminished by heating at 56°C for 20 min.

*Density gradients for localization of collagenase associated with cellular organelles*

Recovery and reproducibility of determinations of succinate dehydrogenase, $Na^+ + K^+$-activated adenosine triphosphatase and glucose 6-phosphatase in gradients was excellent. The distribution and recovery of collagenase was, however, variable. Collagenase activity ranged from the high-density sucrose cushion to the top of the gradient; there was no consistent pattern of localization. In addition, recovery of collagenase applied to various gradients ranged from 6 to 76%.

The amount of collagenase precipitated with cellular fractions in the centrifuge varied, and depended on the method used to prepare the cells. If cells were dissected carefully from the tumour mass, centrifuged, washed twice and then broken by repeated freeze–thawing, the amount of collagenase found associated with particulate fractions was 7–24% of the original activity in the suspension of tumour cells. In contrast, if large masses of tumour and stroma were homogenized vigorously in a Teflon–glass homogenizer, 70% of the total enzyme activity recovered was found associated with the particulate sedimentable fraction. It seemed likely that rapidly sedimenting collagenase represented enzyme associated with collagen fibrils. An additional experiment compared the collagenase and collagen content in the pellet and supernatant (after centrifugation at 9000*g*) of both a crude homogenate of tumour and a careful cell suspension of tumour cells dissected free from large amounts of stroma. In the pellet of the crude homogenate there was 8.8 mg of collagen (94% of the total) and 4756 units of collagenase (84% of the total). The supernatant contained 0.55 mg of collagen and 932 units of collagenase. In contrast, the pellet of the cells prepared with more care to exclude stromal tissue contained only 0.62 mg of collagen (69% of the total) and 580 units of collagenase (12% of the total); in the supernatant portion there were 0.26 mg of collagen and 3980 units of collagenase.

The following experiment was performed to determine whether, as suggested by the above data,

fibrillar collagen added to cell homogenates would bind collagenase. A cell suspension was prepared as described above, washed twice and incubated with 1mM-$NaHCO_3$ (pH8.0)–5mM-$CaCl_2$, sedimented at 27000*g*, resuspended in 0.01M-Tris–HCl (pH7.6)–0.15M-NaCl–5mM-$CaCl_2$, and cells were ruptured with 20 strokes of a Dounce glass homogenizer. The resulting homogenate was separated equally into four portions in the following solutions: (A) 0.01M-Tris–HCl (pH7.6)–0.15M-NaCl–5mM-$CaCl_2$ (0.01M-Tris–NaCl–$CaCl_2$ buffer); (B) 0.25M-sucrose in 0.01M-Tris–NaCl–$CaCl_2$ buffer; (C) 0.40M-sucrose in 0.01M-Tris–NaCl–$CaCl_2$ buffer; (D) 0.01M-Tris–NaCl–$CaCl_2$ buffer plus 10mg of purified insoluble fibrillar guinea-pig skin collagen. After incubation for 20min at 25°C, each sample was centrifuged at 15000*g*, and enzyme activities were measured in the pellet and supernatant solutions (Table 4). Results for all four enzyme activities measured were similar, except for collagenase in the sample to which collagen fibrils had been added; there was approximately one-half the initial activity present in the supernatant after collagen fibrils had been added to the suspension.

## Discussion

Preliminary studies (Harris *et al.*, 1972) showed that most of the collagenase activity recovered from homogenates of rabbit tumour was associated with a pellet containing, among other components, large amounts of 5′-nucleotidase and hydroxyproline; it therefore seemed probable that the collagenase was bound to cell membranes or had been adsorbed on fibrillar substrate. The results of the present study point to its extracellular location on collagen fibrils, and provide no support for the hypothesis that active collagenase is associated with a membranous component of tumour cells. It is probable that, at the time of homogenization, the collagenase is already extracellular, released either by tumour cells or by host cells as part of the 'desmoplastic' or proliferative response of mesenchymal tissues at the invading margins of the tumour. No polymorphonuclear leucocytes were seen in sections of the tumour, and it is unlikely that significant amounts of enzyme were derived from acute inflammatory cells.

There is no evidence that the enzyme characterized here is different from the collagenase produced by monolayer cultures of rabbit synovial cells *in vitro* (Werb & Burleigh, 1974). The specific antibody to the synovial-cell collagenase forms a single precipitin line of identity with purified rabbit synovial collagenase and the crude extracts from our rabbit tumours (Werb & Reynolds, 1975).

It is unusual to find large amounts of collagenase in extracts of tissue homogenates. From single tumours (30–50g) there was sufficient collagenase to degrade 250mg of reconstituted collagen fibrils/h (Harris *et al.*, 1972). How can the large amounts of enzyme found be assimilated with current concepts of collagenase synthesis, release and control of activity? There is mounting evidence in some experimental systems for the existence of inactive precursors of collagenase (Harper *et al.*, 1971; Vaes, 1972; Kruze & Wojtecka, 1972; Oronsky *et al.*, 1973). It is possible that tumour cells activate a zymogen of collagenase already present in the extracellular space, perhaps adsorbed on the fibrils. Reddick *et al.* (1973) and Montfort & Perez-Tamayo (1975) reported results from immunofluorescence studies with antibodies specific for collagenases and showed enzyme to be present on extracellular collagen fibrils at times when no obvious collagen breakdown is occurring.

Another mechanism for development of large amounts of collagenases *in vivo* may be that products of the tumour, or the host reaction to it, may inactivate inhibitors of collagenase present in serum and the tissues. $\alpha_2$-Macroglobulin is recognized as the principal circulating inhibitor of proteinases, including collagenases (Barrett & Starkey, 1973; Harpel, 1973; Werb *et al.*, 1974). In the present studies, a different inhibitor of collagenase was identified and partially characterized; its presence was responsible for much of the 18-fold increase in total collagenase activity recovered in the assay. The inhibitor had a mol.wt. of about 50000 and the reversibility makes it unlikely that it is $\alpha_2$M. Our data also suggest that it is not $\alpha_1$-trypsin inhibitor ($\alpha_1$-AT), because the column fractions containing $\alpha_1$-AT (detected by immunodiffusion) had no capacity to inhibit collagenase, and the fractions with peak collagenase inhibitory substance were not precipitated with anti-($\alpha_1$-AT). There is a precedent for a reversible inhibitor present in tissue extracts of mammalian collagenases (Eisen *et al.*, 1971; Fullmer *et al.*, 1972), and Abe & Nagai (1973) demonstrated that even the inhibition by $\alpha_2$M could be reversed if the $\alpha_2$M itself could be denatured.

Although enzymic activity in the tumour homogenates was substantial, the total protein in each preparation was very small. It seems unlikely from the purification data that there could be more than 1mg of collagenase protein in every 30g of soluble extract. It is likely, however, that a specific collagenase is the rate-limiting enzyme in most of the situations in which collagen is being broken down extracellularly (Harris & Krane, 1974*a*,*b*,*c*). The present results show that this tumour-associated enzyme can cleave $[\alpha 1(I)]_2\alpha_2$, $[\alpha 1(II)]_3$ and $[\alpha 1(III)]_3$ collagens. The site of cleavage of the $\alpha 1(I)$ chain by this and the tadpole enzyme has been identified (Gross *et al.*, 1974). With the chick peptide $\alpha 1$-CB7 (268 residues) as substrate, the rabbit tumour-associated collagenase cleaved between a Gly–Ile peptide bond {772–773 from the *N*-terminus of peptide $\alpha 1[(I)]$}. It is note-

worthy that α1-CB8, a peptide of 279 residues that also contains a Gly–Ile peptide bond, was not cleaved. The minimum sequence of residues sufficient to serve as substrate of purified collagenases is not known, but should be amenable for study now that the entire sequence in this region is known (Fietzek *et al.*, 1973). After cleavage here, significant degradation of the denatured reaction products occurs. Neutral extracellular proteinases and/or intracellular lysosomal enzymes are presumably responsible for further degradation of the collagen fragments. We suggest that many mammalian collagenases may have a similar specificity, and, if sufficiently purified, can be separated from all significant proteinase activity that gives degradation products smaller than $\alpha^A$ or $\alpha^B$.

As a case in point, Woolley *et al.* (1975) have purified rheumatoid synovial collagenase and report that their preparation cleaves $\alpha^A$ and $\alpha^B$ in the form of gelatin to smaller fragments. However, the specific activity of their enzyme with reconstituted fibrils at 37°C as substrate was 312 μg of collagen degraded/min per mg of enzyme protein. Our purified preparation, with the same assay method and units, has a specific activity of 3583 μg of collagen degraded/min per mg of enzyme protein and does not significantly attack denatured $\alpha^A$ or $\alpha^B$. It is likely that even a faint trace of neutral proteinase in a collagenase preparation would be sufficient to degrade gelatin to small fragments.

It is noteworthy that a very pure enzyme preparation was stable up to 1 year at −20°C, because partially purified collagenase from this tumour and from other sources examined in our laboratory become progressively unstable during purification, reaching maximum instability when almost pure.

There has been controversy in the literature on collagenases as to whether the inhibition by EDTA or other chelating agents is reversible if $Ca^{2+}$ ions are subsequently added in excess. Preparation of the $Ca^{2+}$-free enzyme demonstrated that activity was immediately restored on the addition of $Ca^{2+}$ and that, in a $Ca^{2+}$-free inactive state, the enzyme was only slightly less stable than untreated enzyme. It is thought likely (Berman & Manabe, 1973) that the mammalian collagenases are similar to the bacterial collagenase (Seifter & Harper, 1970) and contain zinc as an integral part of the structure. Our studies show that only $Ca^{2+}$ restores significant activity to enzyme inactivated by chelation, and that its presence is essential for collagenolysis. The response to adding $Ca^{2+}$ was immediate, but the percentage of original activity restored was directly related to $Ca^{2+}$ concentration; 1 mM-$Ca^{2+}$ was the minimum concentration needed for restoration of full activity. It is possible that treatment of the collagenase with chelating resins was insufficient to remove $Zn^{2+}$ ions that may be bound in complex fashion within the enzyme molecule.

Determination of the specific activity of mammalian collagenases has been complicated by the lack of an assay that accurately reflects cleavage of each triple-helical molecule at the specific site. Use of the pH-stat requires large amounts of substrate (3 mol of $H^+$ released/mol of collagen) and, unless completely pure enzyme is used, the reaction must be run at temperatures ≤27°C so as to avoid titrating acid released by hydrolysis of peptide bonds elsewhere in the molecule after the initial reaction products are thermally denatured at 32°C ($\alpha^A$) and 29°C ($\alpha^B$) (Sakai & Gross, 1967). The radioactive-fibril assay, although excellent for the assay of large numbers of samples for collagenolytic activity, is too complex for the determination of initial rates of cleavage at one site, since only those molecules at the surface of the reconstituted gels are cleaved by the enzyme (Harris & Farrell, 1972). In addition, unless very pure enzyme is used the presence of non-collagenolytic proteinases may influence the rate of fibril solubilization (Lazarus *et al.*, 1972; Woessner & Ryan, 1973). Substances without proteinase activity [e.g. heparin (Sakamoto *et al.*, 1973), lysosome (Sakamoto *et al.*, 1974) and immunoglobulin G (McCroskery & Harris, 1973)] can affect rates of fibril lysis by collagenase, whereas they have no effect on molecules in solution.

The use of viscometry for the determination of initial rates of collagenolysis is acceptable if measurements can be obtained early enough to permit extrapolation to initial velocity of the reaction. We have used 35°C, a temperature at which cleaved products ($\alpha^A$, $\beta^A$ and $\alpha^B$) make a negligible contribution to viscosity of the solution, yet at which there is negligible thermal denaturation of intact collagen. L-Arginine, added to the reaction mixture, prevents fibril formation (Gross & Kirk, 1958) yet does not affect rates of collagen degradation (McCroskery & Harris, 1973). By using $^{14}C$-labelled collagen and the electrophoreses of reaction products on polyacrylamide gels, with subsequent counting of radioactivity in the bands consistent with intact and $\alpha^A$, $\beta^A$ or $\alpha^B$ pieces, we were able to confirm the validity of this concept; in the experiments with $Ca^{2+}$-free enzyme reported in the present paper there was excellent correlation between the percentage of collagen degraded (measured viscometrically) and $^{14}C$ radioactivity in gel slices. We suggest that the determination of the initial velocity of degradation of collagen by specific collagenase, for use in determining specific activity, be performed by using this method in the viscometer at 35°C.

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