Effects of Lysosomal Collagenolytic Enzymes, Anti-inflammatory Drugs and other Substances on some Properties of Insoluble Collagen

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1. An enzyme system present in a rat liver lysosome-rich fraction was found to liberate soluble hydroxyproline-containing products from insoluble collagen, with maximum activity at pH 3.45. It was concluded that a form of cathepsin D was involved since synthetic substrates specific for trypsin were not hydrolysed. Collagenolysis was enhanced by thiol compounds and inhibited by Cu^{2+} ions and the anti-inflammatory drugs phenylbutazone and ibufenac. 2. The possibility that behaviour of collagen and collagenolysis were modified by various substances, either by destruction of intramolecular and intermolecular bonds in tropocollagen or by electrostatic interactions, is discussed. Insoluble collagen was found to bind electrostatically to chondromucoprotein. This interaction was inhibited by some anti-inflammatory drugs. 3. Possible roles of the lysosomal collagenolytic enzyme system in experimental lathyrism in rats given penicillamine, and in erosion of cartilage in rheumatoid arthritis, are considered. 4. Collagenolysis *in vivo*, which may depend on complex interrelationships between collagen, chondromucoprotein and metal ions, is discussed in relation to possible effects, both harmful and beneficial, of anti-inflammatory drugs used in rheumatoid arthritis.

Most mammalian collagen is relatively inert (Neuberger, Perrone & Slack, 1951), although soluble forms exist which appear to be synthesized and degraded rapidly (Harkness, Marko, Muir & Neuberger, 1954) before undergoing cross-linking reactions to form insoluble collagen (Lindstedt & Prockop, 1961). Under certain circumstances, the turnover of insoluble collagen is considerably increased, as in granuloma tissue elicited by subcutaneous injection of carrageenin (Jackson, 1957), in the post-partum uterus (Woessner, 1962) and in inflamed tissue at a time when large numbers of granulocytic leucocytes are present (Delauney & Bazin, 1965). Little is known, however, of mammalian enzyme systems that bring about this conversion.

Collagenases, acting only at neutral pH and above and probably of non-lysosomal origin, are present extracellularly in cultures of human skin (Eisen, Jeffrey & Gross, 1968), the involuting post-partum uterus (Jeffrey & Gross, 1967), bone cells (Woods & Nichols, 1965) and in synovial tissue in rheumatoid arthritis (Evanston, Jeffrey & Krane, 1967). These enzymes, some of which split tropocollagen across the three α -chains to form three-quarter- and one-quarter-length fragments, may be considered true collagenases. They are rapidly released from cells and have not been detected in tissue extracts,

although a collagenase has been extracted from human leucocyte granules (Lazarus, Brown, Daniels & Fullmer, 1968). Collagenolytic enzymes, with optimum activity at acid pH values and probably of lysosomal origin, are present in the involuting uterus (Woessner & Brewer, 1963) and in rat liver lysosomes (Frankland & Wynn, 1962).

Except for a report by Wynn (1967) on the collagenolytic activity of a rat liver extract, the above workers employed soluble collagen as substrate. It is more relevant, particularly in inflammation, to study the degradation of insoluble collagen, this being the naturally occurring form responsible *in vivo* for the biochemical and biophysical properties of cartilage and other connective tissue. We therefore investigated collagenolysis caused by a lysosome-rich fraction from rat liver, with insoluble collagen as the substrate.

MATERIALS AND METHODS

Chemicals. Saline solution was 0.9% (w/v) NaCl in water. The sources of drugs investigated and methods adopted to dissolve them were given by Anderson (1968). Sodium salicylate and L-cysteine hydrochloride were bought from British Drug Houses Ltd. (Poole, Dorset). Collagen, L-4-hydroxyproline, N^{α} -benzoyl-DL-arginine β -naphthylamide hydrochloride and toluene-*p*-sulphonyl-L-arginine methyl ester were bought from Koch-Light

Laboratories Ltd. (Colnbrook, Bucks.). Collagen was an extract from bovine Achilles tendon of a purity suitable for estimation of collagenase. The fibres were cut finely with scissors to give a coarse powder. Appropriate amounts were suspended in about 4 ml. of saline solution and homogenized in a Potter-Elvehjem homogenizer. The resulting fine suspension was then centrifuged down and traces of soluble protein were removed by washing three times with saline solution, until the final washing contained no material that was precipitated by phosphotungstic acid (5% w/v, in 2M-HCl). The washed insoluble collagen was then suspended in the appropriate volume of saline solution. This treatment gave a collagen suspension from which representative samples could be pipetted. Chondromucoprotein was fraction A (containing 46% of protein and 8.6% of hexuronic acid) isolated from human cartilage (Anderson, 1962). Solutions of chondromucoprotein were prepared as described by Anderson & Lack (1964). In experiments with the hydrochlorides of D-penicillamine and L-cysteine, these compounds were dissolved in the appropriate buffer and the pH of the solution was adjusted to that of the original buffer with 1M-NaOH.

Enzymes. A rat liver granular fraction (lysosomal-mitochondrial fraction), prepared as described by Anderson (1968), was the source of lysosomal enzymes. The appropriate amount of the granular fraction was suspended in saline solution, incubated at 37° for 30 min. and centrifuged. The clear supernatant fluid, containing 66% by weight of the fraction, was used in enzyme experiments. Pepsin (three-times crystallized, from hog stomach mucosa) was bought from Koch-Light Laboratories Ltd. and trypsin (Tryptar) was bought from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex).

Analytical methods. Hexuronic acid was determined by the carbazole method, as modified by Bitter & Ewins (1961), with glucuronic acid as the standard. Hydroxyproline was determined by the method of Woessner (1961), after hydrolysis of the sample in 6M-HCl for 3 hr. at 100° in tightly stoppered tubes. Hydroxyproline was used as the standard. Under these conditions, hydrolysis curves showed 83% release of hydroxyproline. Determination of hydrolysis of benzoyl-DL-arginine β -naphthylamide in the range pH 2.35–7.0, with 4 mg. of the granular fraction, was carried out by the procedure of Ali, Evans, Stainthorpe & Lack (1967). Hydrolysis of toluene-*p*-sulphonyl-L-arginine methyl ester was carried out by using a formol titration method. The substrate (62 mg.) was incubated at 37° with 2.7 mg. of the granular fraction in a total volume of 6 ml. in the presence of 0.07M-sodium acetate buffer in the range pH 3.5–5.0. Samples (1 ml.) were removed at intervals. Phenol Red was added, and each solution was titrated to a light-pink colour with 0.01M-NaOH. Formaldehyde (37%, v/v, at pH 8) was then added (1 ml.), and the mixture titrated back to a light-pink colour with 0.01M-NaOH, this titre being proportional to the extent of enzymic hydrolysis. By using these methods, control experiments showed that both synthetic compounds were substrates for trypsin.

EXPERIMENTAL AND RESULTS

Collagenolytic activity of a rat liver granular fraction

Experimental details are contained in the legends to the figures.

Effect of pH. The collagenolytic activity of the

Fig. 1. Effect of pH and various enzymes on the breakdown of insoluble collagen. To 4 mg. of the rat liver granular fraction, dissolved in 0.25 ml. of saline solution, was added 0.75 ml. of buffer. This was followed by 5 mg. of insoluble collagen (containing 0.7 mg. of bound hydroxyproline) suspended in 0.5 ml. of saline solution. The buffers employed were sodium formate (pH 2.35–3.6), sodium acetate (pH 3.35–5.9) and sodium phosphate (pH 7.0–8.6) at concentrations of 0.2M. Collagen, in the absence of granular fraction, had negligible solubility in the pH range investigated. The tubes were incubated at 37° for 3 hr. Undigested collagen was then centrifuged down, and to 1 ml. of each resulting clear supernatant fluid was added 1.5 ml. of 10M-HCl. After being heated at 100° for 3 hr., the hydrolysates were neutralized and free hydroxyproline was determined. ○, Digestion, expressed as the percentage of total bound hydroxyproline released in soluble form from 5 mg. of insoluble collagen. The collagenolytic activities of 0.5 mg. of pepsin (Δ) and of trypsin (□) under identical conditions are also shown.

Fig. 2. Collagenolytic activity of a rat liver granular fraction in the absence (○) and presence (Δ) of 8 mM-L-cysteine, added as a solution in 0.75 ml. of 0.2M-sodium acetate buffer, pH 3.45. Experimental details were similar to those described in the legend to Fig. 1, except that increasing amounts of the granular fraction (0–4 mg.), dissolved in 0.25 ml. of saline solution, were used.

rat liver granular fraction over the range pH 2.35–8.6 is shown in Fig. 1. Maximum collagenolysis was obtained at pH 3.45. The insoluble collagen was

not a substrate for trypsin over the range pH 6.0–8.6, but was extensively degraded by pepsin, maximum enzyme activity occurring below pH 2.

Fig. 3. Comparison of the effects of D-penicillamine and L-cysteine on the collagenolytic activity of a rat liver granular fraction at pH 3.45. Experimental details were similar to those described in the legend to Fig. 1, except that 0.5 mg. of granular fraction, dissolved in 0.25 ml. of saline solution, was employed. Increasing amounts of L-cysteine (○) and D-penicillamine (Δ) were added to the enzyme mixture as solutions in 0.75 ml. of 0.2 M-sodium acetate buffer, pH 3.45.

Fig. 4. Inhibition by metal ions of the collagenolytic activity of a rat liver granular fraction at pH 3.45. To a series of tubes containing 0.5 mg. of the granular fraction, dissolved in 0.25 ml. of saline solution, were added increasing amounts of CuSO₄ or ZnSO₄, dissolved in 0.75 ml. of 0.2 M-sodium acetate buffer, pH 3.45. To each tube was added 5 mg. of collagen suspended in 0.5 ml. of saline solution. The final concentration of metal ions ranged from 0.001 to 10 mm. A control tube contained the same ingredients, except that metal ions were omitted. The tubes were incubated at 37° for 3 hr.; subsequent experimental details were identical with those described in the legend to Fig. 1. Results were expressed as percentage decrease in the release of soluble hydroxyproline-containing products in the presence of Cu²⁺ ions (○) and Zn²⁺ ions (Δ), compared with the control.

Substrate specificity. The granular fraction did not hydrolyse the trypsin substrates benzoyl-DL-arginine β-naphthylamide or toluene-*p*-sulphonyl-L-arginine methyl ester.

Activation of collagenolysis by thiol compounds. Increased collagenolysis by the rat liver granular fraction in the presence of cysteine and penicillamine (ββ-dimethylcysteine) is shown in Figs. 2 and 3.

Inhibition of collagenolysis by metal ions and by anti-inflammatory drugs. The inhibition of collagenolytic activity of the rat liver granular fraction by Cu²⁺ and Zn²⁺ ions, added as sulphates, is shown in Fig. 4. The reversal by penicillamine of the collagenolysis-inhibiting activity of 1 mM-Cu²⁺ ions is shown in Fig. 5. The inhibitory effects of phenylbutazone and ibufenac on collagenolysis are shown in Fig. 6. Flufenamic acid, 4-amino-phenazone, aspirin, sodium salicylate and chloro-quine had no inhibitory activity.

Interaction of chondromucoprotein with insoluble collagen

Effect of pH and ionic strength. In a preliminary experiment, a solution of chondromucoprotein was

Fig. 5. Reversal by D-penicillamine of the inhibition by 1 mM-Cu²⁺ ions of the collagenolytic activity of a rat liver granular fraction at pH 3.45. To a series of tubes containing 0.5 mg. of the granular fraction, dissolved in 0.25 ml. of saline solution, was added CuSO₄ dissolved in 0.35 ml. of 0.2 M-sodium acetate buffer, pH 3.45. Increasing amounts of D-penicillamine, dissolved in 0.35 ml. of buffer, were then added, followed by 0.5 mg. of collagen suspended in 0.5 ml. of saline solution. The final concentration of Cu²⁺ ions was 1 mM, and the final concentration of penicillamine ranged from 0 to 10 mM. A control tube contained the same ingredients, except that Cu²⁺ ions and penicillamine were omitted. After incubation at 37° for 3 hr., experimental details were identical with those described in the legend to Fig. 1. Results were expressed as the percentage change in the release of soluble hydroxyproline-containing products in the presence of Cu²⁺ ions and penicillamine, compared with the control.

Fig. 6. Inhibition by anti-inflammatory drugs of the collagenolytic activity of a rat liver granular fraction at pH 3.45. To a series of tubes containing 0.5 mg. of the granular fraction, dissolved in 0.25 ml. of saline solution, was added 0.65 ml. of 0.2 M-sodium acetate buffer, pH 3.45. Increasing amounts of the drug dissolved in 0.1 ml. of ethanol were added, followed by 5 mg. of collagen suspended in 0.5 ml. of saline solution. Final drug concentrations were 1, 2 and 4 mm. A control tube contained the same ingredients, except that 0.1 ml. of solvent was used in place of the drug solution. After the tubes had been incubated at 37° for 3 hr., experimental details were identical with those described in the legend to Fig. 1. Results were expressed as percentage decrease in the release of soluble hydroxyproline-containing products in the presence of phenylbutazone (○) and ibufenac (Δ), compared with the control.

added to a suspension of insoluble collagen at pH 5 in 0.025 M-sodium acetate buffer. Centrifugation of the collagen, and determination of chondromucoprotein remaining in the resulting supernatant fluid, showed that a proportion of the chondromucoprotein had become bound to the insoluble collagen and had sedimented with it. Figs. 7 and 8 show the effect of pH and of Ca^{2+} ion concentration respectively on the formation of chondromucoprotein-collagen complexes.

Effect of anti-inflammatory drugs on the formation of chondromucoprotein-collagen complexes. To a suspension of 2 mg. of collagen in 0.5 ml. of saline solution were added 1 ml. of 0.05 M-sodium acetate buffer, pH 4.85, 0.1 ml. of drug solution and 0.3 mg. of chondromucoprotein dissolved in 0.5 ml. of water. The drugs were at final concentrations of 4 mm. A control tube contained the same ingredients, except that the drug was omitted. After standing

Fig. 7. Effect of pH on interaction between chondromucoprotein and insoluble collagen. To a suspension of 2 mg. of collagen in 0.5 ml. of saline solution was added 1 ml. of 0.05 M buffer, followed by 0.3 mg. of chondromucoprotein (containing 25.8 μg. of bound hexuronic acid), dissolved in 0.5 ml. of water. The buffers employed were KCl-HCl (pH 1.0-1.5), sodium formate (pH 2.65), sodium acetate (pH 3.95-6.0) and sodium phosphate (pH 7.2-8.4). Control tubes contained the same ingredients, except that collagen was omitted. After the tubes had stood at 18° for 1 hr., the contents were centrifuged and hexuronic acid was determined in the supernatant fluids. The amount of hexuronic acid that had sedimented with the insoluble collagen was calculated by difference, thus giving an estimate of chondromucoprotein bound to collagen.

at 18° for 1 hr. the contents were centrifuged and the amount of chondromucoprotein bound to insoluble collagen estimated as described in the legend to Fig. 7. The following drugs inhibited the binding of chondromucoprotein to insoluble collagen, values in parentheses indicating the percentage inhibition: ibufenac, (100%); flufenamic acid (100%); mefenamic acid (100%); phenylbutazone (68%). Chloroquine had no effect.

DISCUSSION

Homogenization of insoluble collagen yielded a finely divided product with a large surface area for interaction with enzymes. Denaturation due to destruction of intramolecular and intermolecular tropocollagen cross-links was minimal, the product being insoluble over the pH range investigated. Further, trypsin did not release soluble hydroxyproline-containing products (Fig. 1), any proteolysis at acid pH values probably resulting from enzyme impurities. Pepsin degraded collagen with maxi-

Fig. 8. Effect of Ca^{2+} ions on the binding of chondromucoprotein to insoluble collagen at pH 4. To a series of tubes containing a suspension of 2mg. of collagen in 0.5ml. of saline solution were added increasing amounts of CaCl_2 dissolved in 1ml. of 0.05M-sodium acetate buffer, pH 4, followed by 0.3mg. of chondromucoprotein dissolved in 0.5ml. of water. Final concentrations of Ca^{2+} ions ranged from 25 to 200mM. A control tube contained the same ingredients, except that CaCl_2 was omitted. After the tubes had stood at 18° for 1 hr., they were treated as described in the legend to Fig. 7. The percentage decrease in binding, compared with the control tube, was then calculated.

mum activity near its optimum pH of 2. Collagen was extensively broken down by the granular fraction of rat liver, its action therefore resembling pepsin rather than trypsin. Pepsin probably 'solubilizes' native insoluble collagen mainly by attacking extra-helical telopeptides cross-linking tropocollagen molecules, attack by trypsin being restricted to telopeptides not involved in cross-links (Drake, Davison, Bump & Schmitt, 1966). The granular fraction lacked collagenases capable of releasing soluble hydroxyproline-containing fragments at pH 7 and above.

Since the granular fraction had optimum activity at pH 3.45 and did not hydrolyse synthetic substrates of trypsin, the possibility that the activity was due to cathepsins A, B, C and E was eliminated. A form of cathepsin D was probably present, like that previously reported in bovine spleen (Press, Porter & Cebra, 1960), rabbit spleen (Lapresle & Webb, 1960), rabbit liver (Barrett, 1966, 1967), human spleen (McMaster & Webb, 1963) and rabbit polymorphonuclear leucocytes, lymphocytes and monocytes (Stefanovic, Webb & Lapresle, 1962). Whether these enzymes, which degrade haemoglobin, albumin or chondromucoprotein in the

range pH 3.0–3.9, are collagenolytic is unknown. Cartilage contains a form of cathepsin B with optimum activity at pH 4.7, but appears deficient in collagenolytic enzymes (Ali *et al.* 1967).

Increased collagenolytic activity of the rat liver granular fraction by thiol compounds (Figs. 2 and 3) may have resulted from activation of the cathepsin by their reducing action on it. Cysteine also increases albumin breakdown by human and rabbit spleen cathepsin D, but has no effect on haemoglobin breakdown by cathepsin D in bovine spleen and rabbit liver.

Cysteine and other substances may modify catheptic activity by altering the structure of the substrate, thus changing its susceptibility to enzymic attack. This concept may be relevant when considering the structure of collagen. Soluble tropocollagen, the triple helix formed intracellularly by linkage of three α polypeptide chains, aggregates extracellularly by cross-linking reactions into insoluble collagen fibres. Several mechanisms have been postulated (Piez, 1968). Aldehyde groups on adjacent tropocollagen molecules, obtained by oxidative deamination of lysyl ϵ -amino groups by an amine oxidase, may undergo aldol condensation, or form Schiff bases with unchanged amino groups. Cross-linkages may also involve carbohydrate residues and metal cations. As discussed below, further aggregation may occur after electrostatic interaction between collagen fibres and chondromucoprotein.

Destruction of intermolecular cross-links in collagen decreases tensile strength and promotes solubility. If this is accompanied by thermal denaturation, which occurs spontaneously at 37°, the helical structure may be lost owing to rupture of intramolecular bonds, this result making collagen more susceptible to enzymic attack. For instance, collagen 'solubilized' by hydroxylamine, or subjected to high temperatures that destroy intermolecular cross-links, is more extensively attacked by trypsin (Hörmann, 1965). Treatment of insoluble collagen with thiol compounds *in vitro* may therefore destroy some intermolecular cross-links, for instance those derived from aldol condensation and Schiff-base formation (Piez, 1968), the resulting collagen being more susceptible to catheptic attack (Figs. 2 and 3).

Conversely, formation of additional cross-links involving metal ions (Malik & Salahuddin, 1963; Adam, Deyl & Rosmus, 1966; Adam, Fietzek, Deyl, Rosmus & Kühn, 1968) may render collagen more resistant to enzymic attack. This would explain the inhibition of collagenolysis by Cu^{2+} ions (Fig. 4), the reversal of inhibition by penicillamine then being due to their removal by chelation (Fig. 5).

Orientation of collagen fibres, and the mechanical properties of cartilage and other connective tissues,

may result from non-covalent binding between collagen and chondromucoprotein, the latter being the naturally occurring form of chondroitin sulphate, in which this mucopolysaccharide is linked covalently to non-collagenous protein (Muir, 1964). Clusters of cationic sites on collagen, capable of interaction with mucopolysaccharide anions, promote the formation of soluble and insoluble complexes with chondroitin sulphate and chondromucoprotein (Disalvo & Schubert, 1966; Mathews & Decker, 1968; Toole & Lowther, 1968).

These workers, however, investigated soluble collagen deficient in intermolecular cross-links. The present study shows that highly cross-linked insoluble collagen also interacts with chondromucoprotein, which probably contributes *in vivo* to the ordering of collagen fibres in cartilage. Effects of pH (Fig. 7) and Ca^{2+} ions (Fig. 8) indicate that these interactions are electrostatic, for reasons discussed elsewhere (Anderson, 1967). Inhibition of complex-formation between chondromucoprotein and collagen by some anionic anti-inflammatory drugs (see the Experimental and Results section) could have resulted from these compounds competing more strongly than carboxyl and sulphate groups on chondromucoprotein for cationic sites on collagen. There is evidence that the binding of these drugs to other proteins (Meyer & Guttman, 1968), particularly serum albumin, is ionic in nature (Keen, 1966; Solomon, Schrogie & Williams, 1968). Decreased collagenolysis in the presence of phenylbutazone and ibufenac (Fig. 6) may have been due to inhibition of the protease. However, these acidic drugs may have interacted electrostatically with cationic groups in collagen, causing either, because of their structure, configurational changes resulting in increased collagen stability, or steric hindrance to approach of the enzyme.

These results may have relevance to certain events *in vivo*. For instance, rats given penicillamine and other thiols develop lathyrism, characterized by tissues rich in soluble collagen and low in tensile strength (Nimni, 1968). Postulated mechanisms include destruction of tropocollagen cross-links, as discussed above, and deficient cross-link formation due to combination of thiols with aldehyde groups in collagen (Harkness, 1968; Nimni, 1968; Piez, 1968). Results of this study suggest non-enzymic cleavage of a limited number of cross-links in collagen by thiols, thus reducing its tensile strength, the resulting collagen being more susceptible to attack by lysosomal cathepsins. As described elsewhere (Anderson, 1968), the physiological pH at the surface of connective-tissue cells and macromolecules *in vivo* may be sufficiently low for significant lysosomal collagenolytic activity.

Copper salts given orally to penicillamine-fed rats reverse symptoms of lathyrism (Kaiser,

Henkin & Kare, 1968), probably by forming inactive chelates with penicillamine in the gastrointestinal tract. This parallels the finding that enhanced collagenolysis caused by penicillamine *in vitro* is reversed by Cu^{2+} ions (Fig. 5).

Lysosomal enzymes may also be involved in rheumatoid arthritis (Anderson, 1968), and a collagenolytic enzyme system resembling the one described in this study, which has properties similar to those of the cathepsin D of blood cells, may contribute to erosion of cartilage. Copper, iron and other metal salts have been found to be present in raised concentrations in synovial membrane and synovial fluid in rheumatoid arthritis (Mowat & Hothersall, 1968; Senator & Muirden, 1968). If localized in lysosomes (Muirden, 1966; Goldfischer, 1967) these ions may be considered harmful if they promote enzyme release from lysosomes, and their removal by chelation with penicillamine, claimed to be of benefit in rheumatoid arthritis (Jaffe, 1968), would be advantageous. However, if copper inhibits collagenolysis *in vivo*, as it does *in vitro*, therapy with penicillamine may actually promote erosion of cartilage.

The beneficial action of acidic anti-inflammatory drugs, such as phenylbutazone, may partly result from their ability to inhibit certain lysosomal enzymes (Anderson, 1968), particularly those that degrade mucopolysaccharides, and to stabilize lysosomal membranes (Tanaka & Iizuka, 1968). However, interference by these drugs with electrostatic interactions between chondromucoprotein and insoluble collagen, as happens *in vitro*, may impair *in vivo* the correct spatial configuration of these cartilage components. This would contribute to the unsatisfactory prophylactic nature of these compounds, in addition to their suppression of synthetic mechanisms (Whitehouse, 1965) and the induction by them, at least in skin, of non-lysosomal collagenolytic enzymes (Houck, Sharma, Patel & Gladner, 1968).

Collagenolysis *in vivo* is a complex process probably depending on activity of lysosomal enzymes and interrelationships between collagen, chondromucoprotein and metal ions. Considerations of pathological collagenolysis, as in lathyrism and rheumatoid arthritis, must also take into account, in addition to action of lysosomal enzymes, possible interactions with administered thiol compounds and anti-rheumatic drugs.

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