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Native Cross-Links in Collagen Fibrils Induce Resistance to Human Synovial Collagenase

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A model system consisting of highly purified lysyl oxidase and reconstituted lathyritic chick bone collagen fibrils was used to study the effect of collagen cross-linking on collagen degradation by mammalian collagenase. The results indicate that synthesis of approx. 0.1 Schiff-base cross-link per collagen molecule results in a 2–3-fold resistance to human synovial collagenase when compared with un-cross-linked controls or samples incubated in the presence of β-aminopropionitrile to inhibit cross-linking. These results confirm previous studies utilizing artificially cross-linked collagens, or collagens isolated as insoluble material after cross-linking *in vivo*, and suggest that increased resistance to collagenase may be one of the earliest effects of cross-linking *in vivo*. The extent of intermolecular cross-linking among collagen fibrils may provide a mechanism for regulating the rate of collagen catabolism relative to synthesis in normal and pathological conditions.

Optimal function of connective tissue requires high tensile strength developed in collagen fibrils as a result of biosynthesis of intermolecular cross-links (Piez, 1968; Tanzer, 1973). Current evidence indicates that the initial reaction in collagen cross-linking occurs after onset of fibril formation (Siegel, 1974) and is catalysed by lysyl oxidase (Pinell & Martin, 1968; Siegel & Martin, 1970; Siegel & Fu, 1976; Siegel, 1976). Subsequent reactions related to cross-linking are non-enzymic and are promoted by the close apposition of reactive groups in the fibril after catalysis by lysyl oxidase (Siegel, 1976; Schiffman & Martin, 1970). Lysyl oxidase facilitates conversion of the ε-amino groups of certain lysine and hydroxylysine residues in the non-helical regions of collagen to the corresponding ε-aldehydes (Tanzer, 1973). These aldehydes then form Schiff bases with the ε-amino groups of either a lysine or hydroxylysine residue in other molecules to form the principal intermolecular cross-links, i.e. N^6–6′-dehydro-5-hydroxylysinonorleucine and N^6–6′-dehydro-5,5′-hydroxylysinohydroxynorleucine.

Previous studies with reconstituted fibrils have suggested that cross-linking may not only be important for optimum function of connective tissue *in vivo* but also may be a principal mechanism regulating the rate of collagen catabolism by mammalian collagenase (Harris & Farrell, 1972). This hypothesis is also supported by studies that correlate aging of collagen with resistance to collagenase (Hamlin & Kohn, 1971; Hamlin *et al.*, 1975; Zwolinski *et al.*, 1976). Although the conclusions drawn from all these studies are in agreement, they do not localize the precise point during cross-linking at which resistance to collagenase develops or determine whether bifunctional Schiff-base cross-links are sufficient to cause this resistance. The use of preparations of highly purified lysyl oxidase provides a means for answering these questions. Precisely known quantities of native cross-links were synthesized in lathyritic collagen fibrils and the relative resistance to collagenolysis in these fibrils as a function of the degree of cross-linking was measured. The data presented here indicate that introduction of as few as 0.1 unreduced (and presumably labile) Schiff-base cross-link per molecule of collagen causes a distinct resistance to collagenolysis. Although this resistance is relative, collagen cross-linking is probably a significant regulatory factor of collagen catabolism *in vivo*.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Materials

The materials and chemicals used were obtained as follows: L-[^{14}C]lysine (224mCi/mmol) from New England Nuclear Corp. (Boston, MA, U.S.A.); partially purified bacterial collagenase, pepsin, soya-bean trypsin inhibitor and Tos-Phe-CH_2Cl (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one; TPCK)-

treated trypsin from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); SDS, acrylamide, ammonium persulphate, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylenediamine from BioRad Corp. (Rockville Centre, NY, U.S.A.); Dulbecco's Modified Eagle's medium (high-glucose formulation), Hanks balanced-salt solution, lactalbumin hydrolysate, foetal bovine serum and dog serum from Grand Island Biological Corp. (Grand Island, NY, U.S.A.).

Preparation of lysyl oxidase

Lysyl oxidase was prepared from 17-day-chick embryonic cartilage as previously described (Siegel & Fu, 1976). The enzyme preparation previously designated 'highly purified lysyl oxidase' was used in all experiments (Siegel & Fu, 1976).

Preparation of collagen substrates and synthesis of collagen cross-links

Chick calvaria bone collagen labelled with [^{14}C]-lysine was prepared as previously described (Siegel, 1976). The collagen specific radioactivity was determined for each batch and varied from 2.87×10^{14} d.p.m./mol to 7.12×10^{14} d.p.m./mol. The specific radioactivity of lysine and hydroxylysine varied similarly and ranged from 1.23×10^{13} d.p.m./mol to 2.84×10^{13} d.p.m./mol for hydroxylysine and 8.04×10^{12} d.p.m./mol to 1.63×10^{13} d.p.m./mol for lysine. Samples of approx. 1 nmol were incubated at 37°C in 6mm × 50mm culture tubes for 1 h at 37°C to promote fibril formation. β-Aminopropionitrile (50μg/ml) was added to some tubes. Highly purified lysyl oxidase (0.4μg) was then added to some tubes and the incubation allowed to proceed for various time periods. After incubation, tubes were either frozen immediately or reduced with $NaBH_4$ and analysed for collagen cross-links by amino acid analysis as previously described (Siegel, 1976). The number of cross-links synthesized per molecule of collagen was calculated from the radioactivity measured on amino acid analysis by assuming that the specific radioactivity of N^6–6'-dehydro-5,5'-hydroxylysinohydroxynorleucine was twice the specific radioactivity of hydroxylysine, and the specific radioactivity of N^6–6'-dehydro-5-hydroxylysinonorleucine was equal to the sum of the specific radioactivities of lysine and hydroxylysine. One set of samples was reduced and analysed for cross-links and a matched set was frozen and shipped to Hanover by Air Express in solid CO_2 for collagenase experiments. After shipment, samples were stored frozen at −70°C.

Assay of susceptibility of collagen substrates to collagenolysis

After slow thawing at 4°C, 70–100μl (approx. 1 nmol) portions of the [^{14}C]collagen fibril suspensions were incubated at 37°C in 6mm × 50mm glass tubes for at least 1 h to ensure stable fibril conformation. The fibrils were centrifuged (20min; 17000 rev./min; 20°C) in a Sorvall RC 2B temperature-controlled centrifuge, with the use of plastic adaptors for the small tubes in an SS34 centrifuge rotor. Pelleted fibrils were washed twice by resuspension in 600μl of 0.05M-Tris/HCl (pH7.6)/0.005M-$CaCl_2$/0.02% NaN_3, and subsequent centrifugation as described above. All washes were saved for determination of total radioactivity. Washed fibrils were resuspended in a mixture of partially purified rheumatoid-synovial collagenase [10–100μl in 0.05M-Tris/HCl (pH7.6)/0.01M-$CaCl_2$/0.2M-NaCl/0.02% NaN_3] with or without carrier collagen [50–105μg of guinea-pig skin collagen in solution in 0.05M-Tris/HCl (pH7.6)/0.15M-NaCl]. The latter was added to some assays to stablize collagenase during prolonged incubation; it was shown to offer no competition for collagen in fibril form, because the collagen in solution was quickly cleaved and the fragments formed were rapidly denatured. The final calcium concentration was adjusted to 0.01M with 100μl of 0.1M-Tris/HCl (pH7.6)/0.03M-$CaCl_2$/0.02% NaN_3. Tubes were incubated at 37°C for prescribed time periods and 200μl supernatants were counted for radioactivity after centrifugation (as described above). The remaining supernatant was carefully removed and fibrils were resuspended in fresh buffer solutions, containing carrier collagen and collagenase, and reincubated at 37°C. In this fashion, supernatant from the same tube would be counted after three to five incubation periods with fresh enzyme. A final reaction mixture for each tube contained bacterial collagenase (100μl, 4mg/ml) to digest totally any remaining fibrillar substrate and enable calculation of the total radioactivity present in fibril form in each tube. All aqueous samples were counted for radioactivity in 10ml of Bray's (1960) solution in a Packard liquid-scintillation spectrometer. Data were calculated as follows: c.p.m. in each 200μl portion were adjusted to d.p.m./total sample (350μl). Data could then be expressed as cumulative percentage lysis of collagen substrate as a function of incubation time and units of collagenase used (e.g. collagenase-hours). One unit of collagenase cleaves 1μg of collagen in fibril form in 1 h at 37°C.

Preparation and characterization of rheumatoid-synovial collagenase

Medium was harvested from rheumatoid-synovial cells dissociated from matrix and cultured as monolayers (Dayer *et al.*, 1976). After cells had proliferated to confluence in Dulbecco's modified Eagle's medium and 10% foetal calf serum, culture was continued in Dulbecco's modified Eagle's medium plus 0.2% lactalbumin hydrolysate (Werb *et al.*, 1977). In pilot studies, crude culture medium activated with Tos-Phe-CH_2Cl-treated trypsin (10μg/ml of culture

medium; room temperature; 30min; followed by soya-bean trypsin inhibitor, 40μg/ml of culture medium) was used. Collagenase was partially purified from culture medium after activation with trypsin as above and concentration by pressure filtration through UM10 membranes (Amicon Corp., Lexington, MA, U.S.A.). Two purification procedures were used, both of which resulted in a collagenase preparation capable of specific cleavage of collagen molecules with little or no non-specific proteinase activity. One preparation was passed through a column (2.6cm × 11cm) of agarose (Bio-Gel A-1·5m; 200–400mesh; BioRad, Rockville Centre, NY, U.S.A.) in 0.1M-Tris/HCl (pH7.6)0/.2M-NaCl/0.005M-$CaCl_2$. This agarose preparation had been found previously (McCroskery *et al.*, 1975) to separate mammalian collagenase from other proteins. This sample could degrade 155μg of collagen fibrils/h per ml at 37°C. The second procedure involved anion-exchange chromatography on DEAE-cellulose DE-52 (Whatman, Clifton, NJ, U.S.A.) in 0.05M-Tris/HCl (pH7.6)/0.05M-NaCl/0.005M-$CaCl_2$/0.02% NaN_3 followed by gel filtration on a 1.5cm × 90cm column of AcA 54 (LKB, Hicksville, NY, U.S.A.). This more potent preparation could degrade 2688μg of collagen fibrils/h per ml at 37°C. Samples were assayed for collagenase by using ^{14}C-labelled reconstituted guinea-pig skin collagen fibrils (Harris *et al.*, 1969).

Characterization of reaction products of collagenolysis

Viscometric assay to test the specificity of collagenase was performed at both 27°C and 35°C (McCroskery *et al.*, 1975). At 27°C the specific reaction products α^A (*N*-terminus 75% of the molecule) and α^B (*C*-terminus 25% of the molecule) remain helical. At 35°C the native substrate remains helical, but the α^A and α^B fragments are denatured to gelatin polypeptides. As gelatin they are susceptible to further degradation by any proteinase contaminating the enzyme preparation. Reactions at each temperature were allowed to proceed until the specific viscosity had decreased to 50% of the original values. After stopping reactions with an excess of EDTA to Ca^{2+} present, reaction products were examined by 7.5% (w/v) polyacrylamide-gel electrophoresis in SDS (Neville, 1971). Collagen was purified from guinea-pig skin by the methods of Glimcher *et al.* (1964).

Results

Characterization of collagenase

The rheumatoid-synovial collagenase used in these experiments was capable of cleaving collagen molecules into specific β^A, α^A and α^B fragments, which represent three-quarters and one-quarter of the original β- and α-chain lengths respectively (Fig. 1). In addition, since both α^A and α^B pieces are denatured to gelatin polypeptides at temperatures about 32°C, the reaction products formed at 35°C indicated that the enzyme preparation used contained little or no non-specific proteinase activity directed against primary sequences in the gelatin fragments. This functionally pure collagenase was used in all experiments with cross-linked fibrils.

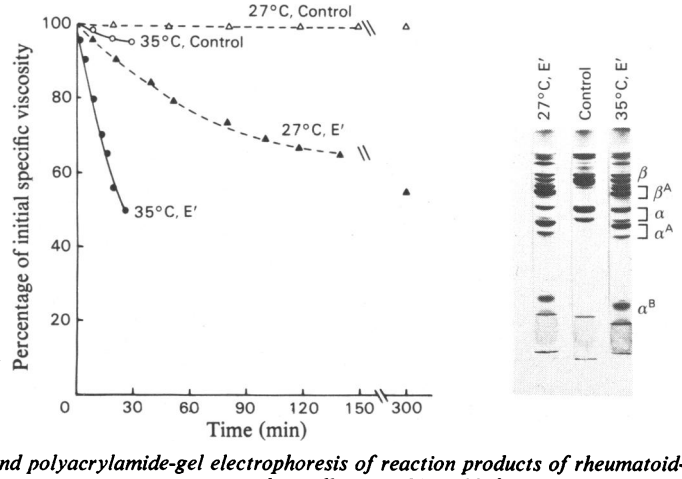

Fig. 1. *Viscosity curves and polyacrylamide-gel electrophoresis of reaction products of rheumatoid-synovial collagenase and guinea-pig skin collagen at 27 and 35°C*

E' is collagenase activated by trypsin. Each assay was stopped by addition of EDTA when viscometric evaluation was completed. The polyacrylamide-gel electrophoresis demonstrated that, although the reaction products (β^A, α^A and α^B) were denatured at 35°C, no cleavage at sites other than the specific one occurred. The doublet bands represent the different migration of the α1- and α2-chains in the skin collagen substrate [$(\alpha 1)_2$ $\alpha 2$].

Table 1. *Effects of β-aminopropionitrile and lysyl oxidase followed by β-aminopropionitrile, on digestion of [^{14}C]collagen fibrils by rheumatiod-synovial collagenase*

For C, [^{14}C]collagen was incubated for 30min at 37°C to form fibrils, then frozen. For β-aminopropionitrile as for C, except β-aminopropionitrile was added after 30min incubation before freezing. For lysyl oxidase+β-aminopropionitrile, [^{14}C]collagen was incubated to form fibrils for 30min, then lysyl oxidase was added for 120min, then β-aminopropionitrile was added and the samples were frozen. After thawing, samples were handled as described in the Experimental section. Each sample contained approx. 1nmol of [^{14}C]collagen with a specific radioactivity of 2.8×10^{14} d.p.m./mol. Collagenase hours = hours of incubation × units of collagenase; 1 unit = 1 μg of reconstituted collagen fibrils degraded/h at 37°C. A = 3.0h×3.9units = 11.7; B = 15.8h×7.8units = 123.2; C = 6.5h×15.5units = 100.8; D = 16.25h×15.5 units = 251.9; E = 3.5h × 15.5 units = 54.3. Cumulative percentage radioactivity released is the sum of soluble radioactivity (d.p.m.) after each subsequent incubation of ^{14}C-labelled fibrils with synovial collagenase divided by the total substrate radioactivity (d.p.m.). Summation of counts was begun after fibrils were washed.

	Radioactivity released (d.p.m.)			Cumulative percentage of radioactivity released		
	C	β-Aminopropionitrile	Lysyl oxidase+β-aminopropionitrile	C	β-Aminopropionitrile	Lysyl oxidase+β-aminopropionitrile
First supernatant	32712	32028	24324	—	—	—
Wash 1	994	1616	2748	—	—	—
Wash 2	606	504	300	—	—	—
Sequential incubation with collagenase						
A	8584	14416	1064	4.1	6.6	0.4
B	8900	8449	3020	8.3	10.5	1.6
C	21678	15276	15376	18.6	17.5	7.8
D	48956	41528	22416	42.0	36.5	16.8
E	14257	18684	12486	48.8	45.1	21.8
Pellet digestion with bacterial collagenase (F)						
	107335	119965	195525			
Total (A–F)	209610	218318	249887			

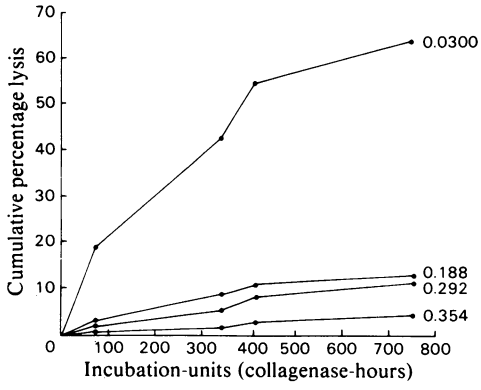

Fig. 2. *Cumulative percentage lysis of control and cross-linked fibrils by synovial collagenase*

The term incubation-units represents cumulative collagenase-hours or the number of hours of incubation at 37°C multiplied by the number of units of collagenase present during that incubation period. Soluble carrier collagen was used in this experiment. The values on the curves are the numbers of cross-links/molecule.

Pilot studies using cross-linked collagen fibrils

^{14}C-labelled fibrils cross-linked with lysyl oxidase were used without reduction by $NaBH_4$ as substrate for collagenase. In general, approx. 10–17% of the total radioactivity in each tube was soluble after the initial thaw and subsequent incubation at 37°C (Table 1). However, less than 1% of total radioactivity was found in supernatant from subsequent washes.

Addition of β-aminopropionitrile (50μg/ml) to collagen to prevent continued (if lysyl oxidase had been added), or spontaneous, cross-link formation had no effect upon rates of collagenolysis. This is shown in Table 1, which also demonstrates that exposure of fibrils to lysyl oxidase (specific number of cross-links/molecule not calculated) significantly retarded the rate of collagenolysis. Addition of EDTA to similar preparations inhibited all solubilization of radioactivity, which is consistent with the information that collagenases are metalloproteinases (Table 1).

Correlation of degree of cross-linking with susceptibility to collagenase

A series of [^{14}C]collagen fibrils was prepared that

had been incubated with lysyl oxidase for variable periods of time, producing cross-link concentrations that varied from 0.03 to 0.35 cross-link/molecule (Fig. 2). The large difference in susceptibility to collagenase between collagen fibrils with 0.030 cross-link/molecule and those with 0.188 cross-link/molecule, shown in Fig. 2, prompted examination of fibrils with intermediary numbers of cross-links.

To rule out any possibility that addition of carrier collagen to the system to provide stability to enzyme over a long period of incubation affected relative rates of lysis, partially purified collagenase was concentrated by pressure dialysis and incubated with fibrils alone for a shorter time period (total duration of assay, 12h) (Fig. 3). A difference in rates of lysis was noted to occur at approx. 0.1 cross-link/molecule, an observation that confirmed a similar previous experiment in which carrier collagen was used (results not shown). There was no significant difference in resistance between fibrils with 0.13 and 0.24 cross-link/molecule. Resistance to degradation induced by cross-links was not absolute at any level; sufficient duration of incubation with a sufficiently large concentration of collagenase degraded even the most highly cross-linked samples. In the experiment shown in Table 2 collagen fibrils that were either weakly or heavily cross-linked were incubated for certain time periods with variable quantities of collagenase. During Expt. A only 2.4% of the radioactivity in the fibrils with 0.59 cross-link/molecule was solubilized; this was very close to background radioactivity. To test whether these fibrils had an absolute or only relative resistance to collagenase, the collagenase-hours were increased greatly by both the amount of enzyme incubated with the fibrils and the time of incubation (Expt. B). The fibrils that were previously completely resistant to collagenase were susceptible to breakdown when exposed to sufficient enzyme for a long enough incubation period. EDTA inhibited all but a trace of breakdown during this long assay (133h) at 37°C.

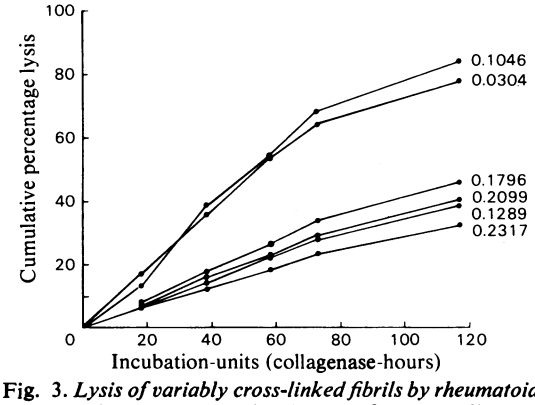

Fig. 3. *Lysis of variably cross-linked fibrils by rheumatoid-synovial collagenase in the absence of carrier collagen*
Specific radioactivity of hydroxylysine was 23 148 000 d.p.m./μmol and that of lysine was 25 584 000 d.p.m./μmol. Cross-links per molecule (the values on the curves) were calculated from the following data:

Incubation time (h)	Dehydrohydroxylysino-norleucine (pmol)	Hydroxylysino-norleucine (pmol)	Total collagen (nmol)	Cross-links per molecule
0	8.98	6.15	0.4973	0.0304
1	30.8	31.9	0.5997	0.1046
2	41.4	35.9	0.5232	0.1289
4	49.9	57.0	0.5951	0.1796
6	46.8	66.0	0.5374	0.2099
24	44.4	83.9	0.5538	0.2317

Study of the mechanism of inhibition of cross-links of collagenolysis

It was decided to determine whether in this model system cross-linking prevented access of collagenase to the catalytic site, or whether hydrolysis could take place but the cleaved fragments were prevented from being freed from the fibril and going into solution. Experiments were designed to approach this question. Control and cross-linked collagen (0.60 cross-link/molecule of collagen) were each incubated with buffer solution or with partially purified collagenase until a substantial portion of the control collagen had been degraded. Specifically 25000c.p.m. more than enzyme blank were present in the 100μl supernatant portion after incubation with rheumatoid-synovial collagenase. The remaining fibrils in each tube were washed twice with water and then solubilized in 0.5M-acetic acid at 4°C for 48h. Under these conditions, more than 95% of the cross-linked collagen is solubilized as α-chains. Electrophoresis of the dissolved washed pellets on SDS/polyacrylamide slab gels revealed no molecular species smaller than α-chains in any sample or molecules of size intermediate between α- and β-chains. These data indicated that cross-linked molecules cleaved by collagenase did not remain bound to the remaining fibrils and therefore insoluble. In additional experiments collagenase-treated cross-linked fibrils were solubilized in acetic acid in the presence of pepsin (1μg of pepsin/100μg of collagen fibrils) overnight at 4°C. Again, SDS/polyacrylamide-gel electrophoresis revealed no products smaller than α-chains or intermediate between α- and β-chains. Incubation of cross-linked fibrils

Table 2. *Collagenolysis of cross-linked fibrils by rheumatoid-synovial collagenase as a function of units of enzyme and time of incubation*

	Expt. A				Expt. B			
Hours of incubation	0.75	2.7	13.0	2.8	20.5	23.6	66.5	22.5
Units of collagenase	15.5	15.5	0.8	17.8	93.0	93.0	46.5	77.5
Collagenase-hours	11.6	41.9	10.4	50.4	1906.5	2194.8	3092.3	1743.8
Cumulative collagenase-hours	11.6	53.5	63.9	114.3	1906.5	4101.3	7193.6	8937.4
Units representing cumulative percentage of collagen degraded								
	Expt. A				Expt. B			
0.218 cross-link/mol of collagen	2.8	8.4	10.8	12.2	—	—	—	—
0.590 cross-link/mol of collagen	0.5	1.1	1.7	2.4	7.2	20.3	57.5	71.5
0.590 cross-link/mol of collagen plus EDTA	—	—	—	—	2.3	3.5	4.6	6.9

simulataneously with both collagenase and trypsin (0.37–3.75 μg/tube) at 37°C did not significantly increase collagenolysis over cross-linked controls incubated with collagenase alone, as determined by measurement of solubilized radioactivity. We have inferred that in this artificial system rheumatoid-synovial collagenase could not gain access to catalytic sites within fibrils that had significant numbers of intermolecular cross-links.

Discussion

Previous studies have shown that introduction of as few as 2.7 methylene-bridge cross-links per molecule of reconstituted collagen fibrils rendered the fibrils insoluble in cold acetic acid and resistant to rheumatoid-synovial collagenase as well as bacterial collagenase (Harris & Farrell, 1972). This effect of artificial cross-linking suggested that the poor solubility and relative resistance to collagenolysis of cartilage collagen fibrils might be related to the presence of intermolecular cross-links (Harris & McCroskery, 1974). This hypothesis was also supported indirectly by a number of other studies showing that suceptibility to collagenase is reduced with aging. Human tendon collagen and collagen from human myocardium are less susceptible to bacterial collagenase with increasing age (Hamlin & Kohn, 1971; Hamlin *et al.*, 1975; Zwolinski *et al.*, 1976). Polymeric insoluble skin collagen is not solubilized by rheumatoid-synovial collagenase capable of completely degrading monomeric collagen (Leibovich & Weiss, 1971). Human insoluble tendon collagen is less susceptible to degradation by purified rheumatoid synovial collagenase than is collagen from soft tissues (Woolley *et al.*, 1975). Finally, when reconstituted calf skin fibrils age *in vitro* for 1 month, they become progressively resistant to solubilization in acetic acid and to cleavage by leucocyte collagenase (Sopata & Dancewicz, 1974). Although all of these studies support the hypothesis that intermolecular cross-linking confers resistance within collagen fibrils to collagenase, interpretation is limited by the possibility that other effects may have been occurring in conjunction with cross-linking. Artificial cross-linking reagents may cause subtle alterations in molecular orientation of substrate which alter collagenase activity. The effect of aging may be due to the synthesis of other molecules such as proteoglycans, which might limit access of collagenase to its substrate. Tissue-specific inhibitors of collagenase might be produced locally. Aging may result in closer molecular packing of collagen molecules in the fibril as a result of improved alignment induced by longitudinal stresses in the fibril.

To minimize factors other than cross-linking that might operate *in vitro* and *in vivo* to inhibit collagenolysis, the present study duplicated conditions *in vitro* as closely as possible to those *in vivo*. This was made possible by the availability of a well-defined model system consisting of highly purified lysyl oxidase and reconstituted collagen fibrils (Siegel, 1976). This two-protein system reproduces the sequence of collagen cross-linking observed in collagen fibrils *in vivo* and allows synthesis of reconstituted collagen fibrils with variable concentrations of cross-links. Although the absolute cross-link content in connective tissue is not readily measurable owing to the formation of non-reducible higher-molecular-weight cross-links, the degree of collagen cross-linking in samples prepared for these experiments was probably low by comparison. The maximum number of aldehyde intermediates formed per molecule of chick calvarium collagen was two (Siegel & Fu, 1976). Whereas normal tissue *in vivo* contains tri- or poly-functional cross-links, our system contained no cross-links that were not reducible and consequently not measurable. The results indicate that the presence of as few as 0.1 Schiff-base cross-link per collagen molecule causes increased resistance to collagenolysis. These cross-

links were not stabilized by reduction before exposure to collagenase and were present as either a Schiff base or the secondary amine derived from the Schiff base by Amadori rearrangement (Eyre & Glimcher, 1973). Since susceptibility to collagenase was measured as the radioactivity soluble after incubation with collagenase, it was necessary to demonstrate that the apparent resistance was not due to retention by cross-linking of cleavage fragments to intact molecules in the fibril. This was shown by destroying the nascent cross-links with acetic acid and examining the products on SDS/polyacrylamide-gel electrophoresis. The electrophoresis studies provided no evidence for collagenolytic activity beyond that observed by measurement of solubilized substrate radioactivity.

In vivo, the two principal conditions in which efficient collagenolysis is required are during periods of rapid growth and remodelling accompanied by biosynthesis of collagen *de novo*, and during degradation of mature cross-linked fibrils. The introduction of small amounts of bifunctional cross-links during biosynthesis *in vivo* may serve primarily to increase collagenase resistance and allow synthesis of stable fibrils during periods of rapid growth and remodelling. Although the resistance to collagenase conferred by cross-linking in this study could be overcome by prolonged incubation, it seems likely that more efficent mechanisms for initial degradation of mature cross-linked fibrils may operate *in vivo*. Support for this inference is provided by the observations that the cross-link region is cleaved to produce soluble monomers *in vitro* from type-I collagen by leucocyte elastase and from type-II collagen by elastase and cathepsin G (Starkey *et al.*, 1977). A neutral proteinase, inhibited by 4-chloromercuribenzoate but not by soya-bean trypsin inhibitor or EDTA, found in sonicates of polymorphonuclear leucocytes in inflamed synovial fluid, was demonstrated to attack non-helical regions of collagen in polymeric form (Steven *et al.*, 1975), and a collagenolytic cathepsin (Etherington, 1976) produced α- from β- and γ-chains at pH 3.5–5.0. If an enzyme such as these were to have access to fibrillar substrate *in vivo* causing removal of the cross-link-containing non-helical telopeptide regions of collagen, it is likely that the monomers generated could then be efficiently degraded by collagenase. Since purified mammalian collagenases have little capacity to cleave native or denatured collagen except at the specific locus three-quarters of the way along from the *N*-terminus (McCroskery *et al.*, 1973; Gross *et al.*, 1974), other enzymes may also be required for efficient collagenolysis in conditions in which mature cross-linked fibrils are being rapidly degraded.

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