In this paper we demonstrate that in the absence of free metal ions, active oxygen species, generated by activated macrophages or xanthine/xanthine oxidase (XOD), carry out oxidative degradation of collagen fibrils type I in conjunction with proteases. The collagen degradation is completely prevented by ascorbate (AH₃) but not by catalase. The free metal ion-independent collagen degradation is a two-step process: (i) oxidation of collagen and (ii) subsequent proteolytic cleavage of the oxidatively modified collagen. AH₃ completely prevents collagen oxidation and thereby protects the collagen from subsequent proteolytic degradation. This is in contrast to free metal ion-catalyzed spontaneous fragmentation of collagen, which is accelerated by AH₃ and inhibited by catalase (Kato, Y., Uchida, K., and Kawakishi, S. (1992) J. Biol. Chem. 267, 23646-23651). Studies using xanthine/XOD and model polypeptides, namely, poly-L-Pro, poly-L-hydroxyproline, poly-L-Lys, and poly(Pro-Gly-Pro) indicate that although O₂ is needed along with metal ions, active oxygen species, generated by xanthine/xanthine oxidase (X/XOD) or stimulated macrophages, cause degradation of collagen (13-15). In this paper, we have used model in vitro systems containing stimulated macrophages as well as X/XOD to investigate the mechanism of oxidative degradation of collagen in the absence of added metal salts. It is known that stimulated macrophages undergo respiratory burst and release in the environment large quantities of O₂ (16-18) and proteinases and collagenases. It is also known that XOD contains metal ions and active oxygen species (19). We present evidence to demonstrate that degradation of collagen involving O₂ is a two-step process: (i) oxidative modification of collagen as evidenced by carbonyl formation and (ii) subsequent rapid degradation of the oxidatively modified collagen by proteinases and collagenses. We further demonstrate that in contrast to acceleration of free metal ion-catalyzed oxidative fragmentation of collagen by AH₃, AH₃ completely prevents free metal ion-independent X/XOD or stimulated macrophage-mediated collagen oxidation and thereby subsequent proteolytic degradation. We have also used model polypeptides, namely, poly-L-proline, poly-L-hydroxyproline, poly(Pro-Gly-Pro), and poly-L-lysine, to elucidate the mechanism of X/XOD-mediated oxidative damage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calf skin collagen type I, XOD grade III from buttermilk, bovine erythrocyte superoxide dismutase, xanthine, phorbol myristate acetate (PMA), ferricytochrome c, AH₃, hydroxypoline, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)) chloride (NBD-Cl), N-(9-fluorenylmethoxycarbonyl) chloride (Fmoc-Cl), trypsin type I, y-chymotrypsin type II, Staphylococcus aureus protease V, collagenase type IA, poly-L-proline (Mₙ > 30,000), poly-L-hydroxyproline (Mₚ 10,000-30,000), poly(PGly-Pro) (Mₚ 2,000-10,000), and poly-L-lysine (Mₚ 30,000-70,000) were purchased from Sigma. Catalase (free of superoxide dismutase) was obtained from CSIR Centre for Biochemicals, New Delhi, India. Cytochrome P450 was purified from liver microsomes of phenobarbital-treated rabbit according to the method of Van Der Hoeven and Van Der Hoeven and Coon using ammonium sulfate precipitation (20). The preparation contained 6.5 nmol of cytochrome P450/mg of protein, assayed by the method of Omura and Sato (21). Desferrioxamine was a gift from Ciba-Geigy, Basel, Switzerland. HPLC solvents were purchased from Qualigen Fine Chemicals, Bombay, India. Phenylisothiocyanate fluoride (PFSF) was a product of Merck. All other reagents were of analytical grade.

**Isolation of Macrophages**—Male guinea pigs (350-400 g) were injected intraperitoneally with 10 ml of sterile mineral oil and exudates harvested 4 days later (22). The cell suspension was washed three times with Earle’s balanced salt solution (22) by centrifugation at 700 x g for 10 min. Erythrocytes were eliminated by hypotonic lysis with 0.2%
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TABLE I

Effects of scavengers of reactive oxygen species on the oxidative degradation of collagen after exposure to activated macrophages and X/XOD

| Addition | Activated macrophage | X/XOD |
|----------|----------------------|-------|
|          | TLC-Fluorometry      | HPLC  |
| Nil      | Nil                  | Nil   |
| 0.1 M xanthine | 91 ± 5              |       |
| 0.1 M glucose | 92 ± 6              |       |
| 0.1 M NaCl | 91 ± 6              |       |
| 0.1 M KCl | 91 ± 6              |       |
| 0.1 M desferrioxamine | 92 ± 6        |       |
| 0.1 M thiourea | 91 ± 6             |       |
| 0.1 M histidine | 91 ± 6          |       |

* SOD, superoxide dismutase.

In the PMA-activated macrophage system, the incubation medium contained 1 mg of calf skin collagen fibrils (~500 nmol of hydroxyproline), 10 μM desferrioxamine in a final volume of 1.5 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 5 mM glucose; incubated at 37°C for 1 h. The amount of O$_2^-$ produced during the initial 5-6 min was 100 ± 10 nmol. Aliquots were made at the following concentrations: macrophages, 1 x 10$^7$ cells; PMA, 1 μg; ascorbic acid, 20 μM; superoxide dismutase, 100 units; catalase, 40 μg; mannositol, 20 μM; thiourea, 10 μM; histidine, 10 μM. Hydroxylproline was estimated by TLC-fluorometry as described under "Experimental Procedures." In the XOXD system, the incubation medium contained 1 mg of calf skin collagen fibrils, 0.9 ml of 0.1 M potassium phosphate buffer, pH 7.4, 10 μM desferrioxamine, 5 x 10$^{-4}$ M xanthine, and 100 millimolars of XOXD in a final volume of 1 ml. Other additions were made as in the macrophage system stated above. The amount of O$_2^-$ produced during the initial 5-6 min was 100 ± 10 nmol. Hydroxylproline contents were estimated both by TLC-fluorometry and HPLC. Results are means of four independent experiments ± S.D.
drosyl radical (‘OH), and singlet oxygen (‘O2) on the degradation of collagen using the TLC-fluorometric technique. As shown in Table I, collagen degradation was completely inhibited by 100 units of superoxide dismutase or 20 μM AH2. Catalase, manniitol, thiourea, and histidine were ineffective, indicating that O2- but not H2O2, ‘OH, or ‘O2 was involved in collagen degradation. Macrophages without activation by PMA, sonicated macrophages + PMA, or cell-free extract of PMA-activated macrophages failed to degrade collagen fibrils.

Degradation of Collagen after Exposure to X/XOD System—Exposure of collagen fibrils to the X/XOD system also resulted in rapid degradation of collagen, as evidenced by the liberation of soluble hydroxyproline-containing peptides (Table I). In this case, hydroxyproline was estimated with both TLC-fluorometry and HPLC. The values were similar. As observed in the case of PMA-activated macrophages, collagen degradation was completely prevented by 100 units of superoxide dismutase or 20 μM AH2. Higher concentrations of AH2 (up to 100 μM) produced similar inhibition. Only collagen + XOD or collagen + xanthine did not cause degradation. It is known that the XOXD system generates not only O2- but also H2O2. However, degradation of collagen exposed to XOXD was not inhibited by catalase. Mannitol, thiourea, and histidine were also ineffective. These results again indicate that O2- and not H2O2, ‘OH, or ‘O2 was involved in oxidative degradation of collagen in the XOXD system.

Role of Iron in Collagen Degradation—Superoxide has been considered to be chemically not that reactive (8), and direct involvement of O2- in protein oxidation has not been substantiated (7). We observed that omission of desferrioxamine from the XOXD system resulted in a 47% increased degradation of collagen over that obtained in the presence of desferrioxamine. The desferrioxamine binding assay (31) indicated the presence of about 1 μM adventitious iron in the reagents and buffers of the incubation mixture and 3.1 μM free contaminating iron in the sample of XOD used. Apparently, this contaminating iron probably caused the increased collagen breakdown in the absence of desferrioxamine. The increase in collagen breakdown in the absence of desferrioxamine was inhibited by catalase but not by AH2. Also, the addition of ADP-Fe3+ to the incubation medium (in the absence of desferrioxamine) resulted in a further increase (90%) in collagen fragmentation. Again, catalase inhibited this increased degradation, but AH2 failed. These results indicate that in the presence of contaminating free iron or added ADP-Fe3+, a fraction of the collagen degradation is mediated by H2O2, as visualized by Stadtman and Oliver (1) in the metal-catalyzed protein oxidation. We consider that oxidative degradation of collagen involving O2- is probably mediated by protein-bound redox iron present in XOD (32) or that secreted by activated phagocytes (33). It is known that the XOD molecule contains four iron centers having redox property (32) and that activated phagocytes secrete cytochrome b558 (33). It is also known that desferrioxamine is incapable of chelating large polypeptide-bound iron (34).

In addition to assessing collagen degradation by measuring the generation of hydroxyproline-containing soluble peptides, degradation of collagen was also evidenced by the production of trichloroacetic acid-soluble fluorescamine-reactive peptides. Fluorescamine reacts with NH2 groups. Production of fluorescamine-reactive materials indicates the production of new NH2-terminal peptides. After exposure of collagen fibrils to the XOXD system, the production of fluorescamine-reactive material continued for 60 min (Fig. 1). In the presence of desferrioxamine, AH2 completely prevented the production of fluorescamine-reactive materials. Catalase, manniitol, thiourea, and histidine were ineffective (data not shown). On the other hand, when ADP-Fe2+ was added to the incubation medium, the production of fluorescamine-reactive material increased about 10-fold, and AH2 failed to prevent ADP-Fe2+-mediated collagen breakdown (Fig. 1). These results confirm that there are two distinct mechanisms of oxidative degradation of collagen: (i) mediation by protein-bound redox iron and completely inhibited by AH2 and (ii) catalysis by free iron ions and stimulated by AH2.

Involvement of both O2- and Proteinases in Collagen Degradation—After the addition of PMA to the macrophage system, O2- production continued only for the initial 5–8 min after which no further O2- was produced. Superoxide production was monitored by the reduction of ferricytochrome c added initially to a separate but identical incubation mixture. When ferricytochrome c was added 10 min after the addition of PMA, no reduction of ferricytochrome c was noticed, indicating that O2- production virtually stopped. Although O2- production stopped, degradation of collagen continued almost linearly for at least 60 min. This indicates that O2- is probably needed initially for oxidative modification of collagen and that the modified collagen is then probably hydrolyzed by proteinases and collagenases released from PMA-activated macrophages (18). This was substantiated by the fact that the addition of inhibitors of proteinases and collagenases, namely, PMSF (100 μM) and EDTA (20 mM), 15 min after the addition of PMA prevented further release of hydroxyproline-containing soluble peptides. Similar observation was made in the XOXD system where PMSF (100 μg) added initially to the incubation mixture almost completely inhibited collagen degradation. PMSF did not inhibit O2- production by XOXD. The proteolytic cleavage in the XOXD system was apparently carried out by contaminating proteinases present in XOD (19). No hydroxyproline was released within the short span of the 1-h incubation period when untreated collagen was incubated with proteinases and collagenases. Once the collagen was oxidatively modified, AH2 or

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**Fig. 1.** Collagen degradation by X/XOD as evidenced by the release of fluorescamine-reactive material. Incubation conditions were as in Tables I and II. After incubation, the mixture was deproteinized with 10% trichloroacetic acid. As mentioned under “Experimental Procedures,” 0.25-ml aliquots of neutralized trichloroacetic acid-soluble supernatants were used for the fluorescamine test. The fluorescamine unit (154) obtained after incubation of collagen fibrils in the absence of xanthine but in the presence of XOD was deducted from all experimental data. Values are the means of three independent determinations; S.D. < 10%. ○, ADP-Fe5+; ▲, ADP-Fe3+; △, in the absence of ADP-Fe5+; x, in the absence of ADP-Fe3+; +, AH2.
superoxide dismutase failed to prevent the degradation of the modified collagen. The results indicate that oxidative degradation of collagen is a two-step process: (i) oxidative modification of collagen, which is inhibited by AH, or superoxide dismutase; and (ii) proteolytic degradation of the oxidatively modified collagen, which is inhibited by protease inhibitors but not by AH<sub>2</sub> or superoxide dismutase.

**Oxidative Modification of Collagen as Evidenced by the Introduction of Carbonyl Groups—Measurement of carbonyl groups is a sensitive assay for assessing oxidative modification of proteins (28). We have estimated carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (28).** Table II shows that treatment of collagen fibrils with X/XOD results in a large increase of hydrazone formation. Since XOD contains contaminating proteinases, we have used PMSF to prevent proteolytic degradation of oxidatively modified protein and thereby loss of carbonyl in the soluble peptides. Hydrazone was not produced when collagen was treated with either xanthine or XOD. Table II further shows that hydrazone formation was completely inhibited by AH<sub>1</sub> or superoxide dismutase but not by catalase. That AH<sub>1</sub> inhibits X/XOD-mediated oxidative modification of protein has also been reported by others (35). However, when ADP-Fe<sup>3+</sup> was added to the incubation medium in the absence of desferrioxamine, collagen oxidation was 74% stimulated by AH<sub>1</sub> indicating that in the presence of ADP-Fe<sup>3+</sup> AH<sub>1</sub> acted as a prooxidant.

Fig. 2 shows that carbonyl formation in collagen is a function of XOD concentration. The amount of carbonyl increases linearly with increased concentration of XOD from 0.1 to 0.5 nmol studied. However, as shown in Table II, XOD alone cannot oxidize collagen. Superoxide is needed along with XOD because X/XOD-initiated collagen oxidation is completely prevented by superoxide dismutase. Moreover, collagen formation virtually stops after 10 min of incubation, when O<sub>2</sub> generation also ceases. Although collagen oxidation is dependent on O<sub>2</sub> formation, the amount of carbonyl formation does not appear to be stoichiometrically related to O<sub>2</sub> concentration, because even with 0.1 nmol of XOD, 20 nmol of O<sub>2</sub> is generated, which is theoretically more than sufficient to produce the maximum amount of carbonyl (2 nmol) formed (Fig. 2).

**Oxidatively Modified Collagen Is Readily Hydrolyzed by Proteinases and Collagenases—**It is known that oxidized proteins are highly susceptible to proteolytic degradation (1–9). This has also been confirmed for collagen by SDS-PAGE of native and oxidatively modified collagen fibrils (Fig. 3). Fig. 3 shows that oxidized collagen isolated after pretreatment with X/XOD undergoes random hydrolysis by trypsin + chymotrypsin, producing a number of low molecular weight peptides ranging from 14,000 to 90,000. Native collagen is not hydrolyzed. Also, no hydrolysis is obtained when X/XOD treatment of collagen fibrils is done in the presence of AH<sub>1</sub> (50 μM), which further confirms that AH<sub>1</sub> prevents oxidative damage of collagen.

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**Table II**

| System                  | Carbonyl groups (hydrazone formed/mg of collagen) | n mole/mg |
|-------------------------|--------------------------------------------------|-----------|
| Collagen                | 0.68 ± 0.09                                      |           |
| Collagen + X            | 0.68 ± 0.09                                      |           |
| Collagen + XOD          | 0.68 ± 0.10                                      |           |
| Collagen + X + XOD      | 2.70 ± 0.15                                      |           |
| Collagen + X + XOD + AH<sub>1</sub> | 0.68 ± 0.09                                  |           |
| Collagen + X + XOD + SOD| 0.68 ± 0.10                                      |           |
| Collagen + X + XOD + catalase | 2.68 ± 0.15                                   |           |

**Fig. 2. Relationships between XOD concentration/O<sub>2</sub> generation and oxidation of collagen.** Carbonyl groups were estimated as described under "Experimental Procedures." The incubation mixture containing desferrioxamine and PMSF was same as in Table II. A basal value of 0.68 ± 0.09 obtained with native collagen was deducted from the experimental data. Carbonyl groups were estimated after 10 min of incubation. Prolonged incubation did not produce further hydrazone formation. Results are the means ± S.D. of four independent determinations; S.D. < 10%.

**Fig. 3. SDS-PAGE of native and oxidized collagen after treatment with proteolytic enzymes.** Native and oxidized collagen (isolated as in Table II) were incubated with trypsin (50 μg) plus chymotrypsin (25 μg) in 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, for 1 h at 37 °C. After incubation, the mixture was centrifuged, sedimented collagen discarded, and the supernatant freeze-dried and dissolved in 50 μl of water. Five μl of this solution was subjected to 12% SDS-PAGE. Lane 1, native collagen treated with trypsin/chymotrypsin; lane 2, oxidized collagen + trypsin/chymotrypsin; lane 3, as in lane 2 except that during treatment with xanthine plus XOD, 50 μM AH<sub>1</sub> was present; lane 4, only trypsin/chymotrypsin. Protein bands were visualized with silver staining (30).
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**Table III**

| Polypeptide          | -AH<sub>2</sub> | +AH<sub>2</sub> |
|----------------------|----------------|----------------|
| Poly-L-Pro           | 1.70 ± 0.12    | Nil            |
| Poly-L-Hydroxyproline| Nil            | Nil            |
| Poly-L-Lys           | 0.82 ± 0.09    | Nil            |
| Poly(P&rsquo;Pro-Gly-Pro) | 1.14 ± 0.08  | Nil            |

**Oxidation of Model Polypeptides**—To elucidate the mechanism of AH<sub>2</sub>-inhibitable collagen oxidation, we have studied oxidation of model polypeptides using X/XOD. The collagen helix contains the characteristic repeating sequence of Gly-X-Y, where X and Y are often Pro and hydroxyproline, respectively. We have studied oxidation of poly-L-Pro, poly-L-hydroxyproline, and the collagen-like sequence, namely, poly(P&rsquo;Pro-Gly-Pro).

Type I collagen contains about 4% Lys (36), so we have also studied oxidation of poly-L-Lys. We have observed that oxidation of poly-L-Pro/poly-L-Lys/poly(P&rsquo;Pro-Gly-Pro) by X/XOD in the presence of desferrioxamine is completely inhibited by AH<sub>2</sub> (Table III). The oxidation was assayed by carbonyl formation.

In contrast to this, when ADP-Fe<sup>3+</sup> was added along with X/XOD in the absence of desferrioxamine, the oxidation of poly-L-Pro was 100% enhanced, and it was further stimulated (125%) by AH<sub>2</sub>. The results again confirm that in the absence of metal ions AH<sub>2</sub> acts as an antioxidant, but in the presence of ADP-Fe<sup>3+</sup> it acts as a prooxidant. We have further observed that in contrast to poly-L-Pro, poly-L-hydroxyproline is not oxidized by X/XOD.

**Oxidation of Model Polypeptides as a Function of the Concentration of Protein-bound Redox Iron**—It has been indicated that although O<sub>2</sub> is needed along with XOD for collagen oxidation, carbonyl formation in collagen is a function of XOD concentration (Fig. 2). This has been substantiated further by our work with poly-L-Pro and poly(P&rsquo;Pro-Gly-Pro). Using X/XOD as a source of O<sub>2</sub> and keeping the amount of O<sub>2</sub> production limited (5–9 nmol) with a limiting concentration of xanthine (25 μM), the formation of carbonyl in poly-L-Pro appears to be a direct function of the concentration of XOD iron. Fig. 4 shows that carbonyl formation in poly-L-Pro increases linearly with increased concentration of XOD iron. Cytochrome P450 is another enzyme that is known to contain protein-bound iron having redox property (37). Fig. 4 further shows that keeping the amounts of XOD (0.4 nmol atom of iron) and O<sub>2</sub> (5 nmol) constant and adding increased amounts of cytochrome P450 in the medium result in a linear increase of carbonyl formation. A similar observation has been made using poly(P&rsquo;Pro-Gly-Pro) in the place of poly-L-Pro (Fig. 5). However, ferritin (20 μM) or hemoglobin (2 μM) could not replace cytochrome P450.

**DISCUSSION**

In the present investigation, we have demonstrated that in the absence of free metal ions active oxygen species, generated by activated macrophages or X/XOD, carry out oxidative degradation of calf skin collagen fibrils in conjunction with proteases. The triple helical structure of collagen, particularly type I, is normally resistant to the action of most proteases. However, once the collagen is oxidatively modified, the fibrils become highly susceptible to proteolytic degradation. This is consistent with the view that oxidatively modified proteins are highly sensitive to proteolytic breakdown (1–9). Although free metal ion-catalyzed oxidation of collagen has provided valuable information about the mechanism of oxidative fragmentation of collagen (11), its relevance to the in vivo situation is questionable, apparently because in the normal physiological condition most of the metal ions are not free and remain tightly bound with proteins. The mechanism of free metal ion-independent collagen oxidation is distinctly different from that catalyzed by Cu(I)/H<sub>2</sub>O.<sub>2</sub>. The free metal ion-independent collagen degradation is a two-step process: (i) oxidation of collagen and (ii) subsequent proteolytic cleavage of the oxidatively modified collagen. In the absence of free metal ions, AH<sub>2</sub> completely prevents oxidation of collagen and thereby protects the collagen from subsequent proteolytic degradation. Catalase is ineffective. This is in contrast to Cu(I)/H<sub>2</sub>O.<sub>2</sub>-catalyzed spontaneous oxidative fragmentation of collagen which is accelerated by AH<sub>2</sub> but inhibited by catalase (11).

It has been shown by Davies and co-workers (7, 9) that O<sub>2</sub> alone, generated by <sup>60</sup>Co radiation, does not significantly damage proteins. We have observed that collagen oxidation by X/XOD is rather a function of protein-bound redox iron of XOD. XOD (EC 1.1.3.22) is a homodimer with a molecular weight of 300,000, having one molybdenum center, two iron-sulfur centers, and one molecule of FAD per subunit (38). It has been reported (38) that during O<sub>2</sub> production (G<sub>6</sub> reduction) the iron-sulfur centers of XOD are transiently reduced in the course of electron transfer from the molybdenum center to the flavin. Redox iron is also probably involved in collagen oxidation by activated macrophages, which are known to secrete in the medium cytochrome b<sub>558</sub> (38). For collagen oxidation, only XOD is ineffective. X/XOD-mediated oxidative modification of collagen is completely inhibited by superoxide dismutase. This indicates that both protein-bound redox iron and O<sub>2</sub> are needed to prod-
Iron of Xanthine Oxidase (n atoms)

- 0.25
- 0.5
- 0.75
- 1.0

Cytochrome P450 (n mole)

- 0.25
- 0.5
- 0.75
- 1.0

Carboxyl groups (mol/mg Protein)

- 0.25
- 0.5
- 0.75
- 1.0

**Fig. 5.** Correlation between XOD-initiated oxidation of poly(Pro-Gly-Pro) and XOD iron or cytochrome P450. Incubation conditions are same as in Fig. 4 except that poly-L-Pro was replaced by 1 mg of poly(Pro-Gly-Pro). Data represent the means of three independent determinations; S.D. < 10%. ○, 25 μM xanthine, 0.1 nmol of XOD and cytochrome P450 (0.25-1 nmol); ◇, 25 μM xanthine plus XOD (0.1-0.5 nmol).

produce an active oxygen species for collagen oxidation. The involvement of redox iron has been substantiated by our work with model polypeptides. Oxidations of poly-L-Pro and poly(Pro-Gly-Pro) have been found to be a direct function of the concentration of XOD iron. Cytochrome P450 also stimulates this oxidation.

If the results obtained in vitro with activated macrophages are applicable to the in vivo situation, then our results may throw some light on the poorly understood mechanism of collagen degradation in scurry and protection by AH₃. The extracellular matrix of mammals contains numerous macromolecules that undergo oxidative burst during phagocytosis and release in the environment large amounts of O₂(16), redox protein like cytochrome b₅₅₇ (33), as well as metalloproteinases and collagenases (18). We have presented evidence that activated macrophages carry out oxidative degradation of collagen and that AH₂ completely prevents this degradation. Superoxide dismutase also prevents it, but in contrast to AH₂ which is ubiquitous in vitro, the content of superoxide dismutase in the extracellular fluid is negligible (39). Only a small amount of glycosylated tetrameric superoxide dismutase is present in the extracellular fluid (40). This imparts a specific important role of AH₂ for the protection of collagen in the extracellular matrix of mammalian tissues.

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