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LONG-TERM EFFECTS OF NEUTRAL PROTEASES ON COLLAGEN DEGRADATION IN THE RABBIT CORNEA

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Abstract

I have investigated the effects of neutral proteases on the collagen in the rabbit cornea. Knowing that matrix metalloproteinanse I (MMP I - collagenase) is the primary source of interstial collagen degradation, we asked what the long term affects were of other neutral proteases on collagen degradation. By exposing 3mm rabbit eye corneal punches to various concentrations of endo and exopeptidases, I have shown that leucine aminopeptidase, carboxypeptidase B, carboxypeptidase A, alpha chymotrypsin (bovine), L-tosylamido-2-phenyl ethyl chlorolmethyl ketone-trypsin, plasmin (human), cathepsin G (human), and elastase (human), all have some degradative effect on interstitial collagen ranging from 0.5% to 13% of the total collagen present in corneal punches in 4 days. These findings suggest that these proteases may have very significant effects on overal all collagen metabolism over extended time periods. By heat denaturing the collagen in corneal punches, I have also shown that 63% to 73% of the total collagen in young corneal punches will solubilize out in neutral buffer, while only 22% to 33% of the total collagen in mature corneal punches is liberated. This finding suggest that collagen crosslinking by nondisulfide bonds is somewhat effected by aging. Finally, by

exposing sections of a 2mm peripheral ring of an 8mm central corneal punch to neutral buffer, I have shown that 1% to 6.6% of the native interstitial collagen will solubilize out of the medial and lateral sections of the ring, but not in the superior and inferior zones. These findings suggest a regional physiological difference on the surface of the eye, possibly in the area where the eyelids meet during blinking.

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Introduction

Collagen types I, II, and III, the interstitial collagens, are the major components of the connective tissue. These structural proteins are each composed of 3 polypeptide (α) chains which together form a triple helix and thus, a rod shaped molecule. The formation of these molecules originates through assembly of pro α chains into a procollagen molecule. Procollagen is secreted into the extracellular matrix, where it is converted to collagen by cleaving the telopeptide ends and other biochemical processes it is converted to collagen.

Type I collagen has a complex structure which has been studied in detail. The type I collagen molecule consists of 2 α 1(I) chains and 1 α 2(I) chain which are aligned and twisted into a triple helical structure. The aligned structures are actually the pro forms of these α chains which consist of 3 major regions; the N-terminal peptide and C-terminal peptide precursors and the α chain. The precursor propeptides are then cleaved off and the alpha chain remains. Piez & Reddi (1984) provides the complete sequence of the α 1(I) chain of calf skin collagen. The α 1(I) chain of calf skin collagen is made up of 3 major regions. The N-terminal telopeptide end contains 16 amino acid residues, which is nontriplet and thus non-helical, as is the C-terminal end, which contains 26 amino acid residues. These nontriplet regions are thus readily susceptible to protease action due to there globular form. The large central region of the chain consists of 338 gly-X-Y triplets where X and Y are occupied by other amino acids. One third of the total X and Y positions are held by proline or hydroxyproline in mammals and almost no other protien contains appretiable amounts of hydroxyproline, making the hydroxyproline assay the assay of preference in the study of degradation. The central region is helical and thus not a highly susceptible substrate for proteases except for matrix metalloprotienase I. The MMP I degradation products of type I collagen denature, as does the molecule itself above 37°C, creating gelatin. This gelatin or denatured collagen then becomes susceptible to most neutral proteases.

Collagen metabolism is mediated by fibroblast cells which secrete both collagen and matrix metalloproteinases (MMPs) to degrade collagen. Collagenase (MMP1) specifically initiates collagen degradation and is secreted in the pro form, as are the other MMPs (Piez and Reddi, 1984). The pro forms of MMPs are activated by endopeptidases, some of which are capable of degrading other matrix components including native interstitial collagen.

Even though MMP1 is the primary source of native interstitial collagen degradation, we asked what the long term effects of other neutral proteases were on collagen degradation. The intent of this report, then is to investigate the degradative effects of various exopeptidases and endopeptidases on interstitial collagen over extended periods of time (days). The exopeptidases studied included leucine aminopeptidase (specific for N-terminal residues with free alpha amino groups especially when leucine), carboxypeptidase A (specific for C-terminal residues unless basic or proline), and carboxypeptidase B (specific for C-terminal lysine or arginine). Specifically we examined the class of endopeptidases referred to as the serine proteases. Endopeptidases are classified best by the essential catalytic group of the enzyme and group specific inhibitors. Thus serine proteases are those proteases whose actions are inhibited by diisopropyl phosphorofluoridate [(Dip-F) 4% v/v in dry propan - 1- ol]. Other group specific inhibitors with broad activity are phenylmethanesulfonylflouride (Pms - F), and lima bean soya bean trypsin inhibitor (100ug/ml). Optimum Ph for this group of proteases is expected in the range of 7 - 9 (Barrett, 1977). These proteases include elastase (specific for peptides adjacent to neutral residues), trypsin (specific for peptides at carboxyl ends involving arginine or lysine), chymotrypsin (specific for peptides at carboxyl groups of aromatic amino acids), plasmin (specificity similar to trypsin), and cathepsin G (unknown specificity) (Dixon, 1964) which degrade various structural glycoproteins and can activate some MMPs (Emonard and Grimaud, 1990).

Preliminary studies in the lab of Dr. Barbara Johnson-Wint indicate that there is a regional aspect to the susceptibility of rabbit corneal collagen to solubilize in neutral buffer solution. It has been

consistently shown that the 2mm peripheral zone of a 8mm central corneal punch liberates a certain amount of collagen during incubation at 37°C , 5% CO_2, 100% humidity in neutral buffer. The work of Lass, Baker, and Klein (1987) showed a significant net increase in total collagen synthesis in the peripheral area, which was significantly greater than that in the central area of grafted rabbit corneas. Thus, a regional approach to the study of the action of the endopeptidases was also a feasible investigation.

This regional investigation may involve the crosslinking of the collagen. This also may have something to do with aging, as it seems that the mature rabbit eye is less susceptible to solubilization after denaturation. This is most likely due to increased crosslinking of the collagen in the mature cornea. It is known that collagen types I and II are extensively crosslinked by nondisulfide covalent bonds. The crosslinks are monomers containing a lysine-derived aldehyde, allysine or dimers joined by allysine aldol. Allysine also forms aldimine condensation products with lysine and hydroxylysine. The aldol crosslinks are intramolecular and the aldimine crosslinks are intermolecular. These crosslinkages enable the molecules to resist the physical forces normally encountered in different locations of the body (Piez & Reddi, 1984). Regional physiological differences may exist on the surface of the eye due to blinking of the eyelids, which are constantly sweeping foreign particles over the cornea and concentrating them and tear growth factors in a horizontal axis. Thus, there could be something biochemically different occurring here which would lead to differences of collagen crosslinking, overall collagen concentration, and susceptibility of that collagen to degradation by proteases.

The rabbit cornea is an excellent and easily obtainable source of collagen and previous work by Lass, Ellison, Wong, and Klein (1986) describe the qualitative collagen degradation in experimental corneal graphs. This shows the potential for the loss of intrinsic collagen from the cornea by protease action.

The preceding issues, including long term effects of endopeptidases, regional susceptibility to degradation or solubilization, and crosslinking of the collagen were all dealt with in

an attempt to better understand the regional chemistry of the collagens in the rabbit cornea and possible non-MMP1 long-term (days, months, years) mechanisms of collagen turnover.

Materials & Methods

Chemicals

Leucine aminopeptidase, carboxypeptidase A, carboxypeptidase B, alpha chymotrypsin (bovine), and L-tosylamido-2-phenyl ethyl chloromethyl ketone-trypsin (TPCK-trypsin) were from Worthington Biochemical Corporation. Plasmin (human), cathepsin G (human), and elastase (human) were from Calbiochem. Protease inhibitor set was from Boehringer Mannheim. Calcium chloride, zinc chloride, sodium azide, citric acid, cis-4-hydroxy-L-proline, and chloramine T were from Sigma. Sodium chloride, dimethylamino-benzaldehyde (DMBA), and 60% perchloric acid were from Mallinckrodt. Sodium acetate, sodium citrate, and hydrochloric acid were from Fisher Scientific. Isopropyl alcohol was from Baxter. Deionized water (QH_2O) was obtained from a Milli-Q-Water System, made up of 2 deionizing cartridges, one carbon cartridge, and a 5000MW cut-off membrane.

Corneal Tissue

The source of corneal tissue for these experiments was whole young and mature rabbit eyes from Pel-Freez Biologicals. The corneas obtained from the mature rabbit eyes averaged 16mm in diameter while the corneas obtained from the young rabbit eyes averaged 13mm in diameter. These eyes were stored at -4°C and thawed as needed.

Dissection and Preparation of Tissues

When needed, eyes were thawed in 50ml Hank's Balanced Salt Solution (HBSS) in a 100mm Falcon tissue culture plate. Using sterile forceps to stabilize the eye, a sterile scalpel was used to remove the cornea by cutting through the sclera immediately adjacent to the cornea. The iris was then peeled from the back of the cornea and the cornea was laid as flat as possible on the culture plate cover. Small incisions in the surrounding sclera were made at the 12 o'clock position (the largest muscle insertion) 3, 6, and 9 o'clock positions to further flatten the naturally convex cornea. A 3mm punch cutting tool was then utilized to remove 3mm buttons from the edge, center,

and the intermediate zone of the cornea (FIG. 1). If the punches of cornea were to be denatured each was placed in a 10ml test tube with a 0.5ml buffer C solution (50mM Tris · HCl, pH 8, 0.15M NaCl, 1.0mM $CaCl_2$, 1.0uM $ZnCl_2$, 0.05% NaN_3 + 0.1% triton X-100) and placed in a beaker of boiling water for 5 minutes. To determine fragment size of denatured collagen a sample was taken after boiling, and after dialysis against water in a 3500 molecular weight cut-off dialysis tubing. To inhibit the action of all known classes of intrinsic proteases which could possibly degrade denatured collagen, a protease inhibitor "cocktail" was added prior to boiling, containing: antipain-dihydrochloride (5mg/ml), 4 - amidinophenyl-methanesulfonylflouride [APMSF (2mg/ml)], aprotinin (1mg/ml), bestatin (4mg/ml), chymostatin (10mg/ml), E-64 (10mg/ml), 1-10 phenanthroline (1mM), leupeptin (0.05mg/ml), pepstatin (0.07mg/ml), and phosphoramidon (3mg/ml). If the punches were not to be denatured they were either used immediately or collected and stored in a micro-centrifuge tube containing buffer C. In a later experiment to verify the solubilization of collagen from a 2mm peripheral ring of a 8mm central punch, these punches were taken (8mm central and then a 6mm central of that). The 2mm ring was then cut (in reference to the largest muscle insertion as superior) into superior, inferior, lateral, and medial sections. Each section was immediately placed in buffer C and incubated for 4 days.

Enzyme Assays

Each 3mm corneal punch was placed into a well of a 24 well Costar tissue culture plate with 0.5ml of buffer C or protease solution. Up to eight animals (four young and four mature) were assayed at a time producing up to four replicates for each corneal region of each animal. The neutral proteases used were a three peptidase solution: leucine aminopeptidase [(1.1units/ml),1 unit hydrolyzes 1 umol of L-leucinamide per minute at 25°C, pH 8.5] carboxypeptidase B [(1.4units/ml), 1 unit hydrolyzes 1 umol of hippuryl-L-arginine per minute at 25°C, pH 7.65] and carboxypeptidase A [(1.1units/ml) 1unit hydrolyzes 1 umol of hippuryl-L-phenylalanine per minute at 25°C, pH 7.5]. Also used independently were; TPCK-trypsin (0.02%),

elastase [human (40units/ml - 1 unit hydrolyzes 1 umol of MeO-Suc-Ala-Pro-Val-PNA per minute at 25°C, pH 8)], cathepsin G [human (4.4units/ml - 1 unit hydrolyzes 1 umol of Suc-Ala-Ala-Pro-Phe-PNA per minute at 25°C, pH 7.5)], plasmin [human (1unit/ml - 1 unit hydrolyzes 1 umol of tosyl-Gly-Pro-Lys-PNA per minute at 25°C, pH 7.8)] and alpha chymotrypsin [bovine (0.02%)]. These plates were incubated at 37° C, 5% CO_2, 100% humidity in a tissue culture incubator. The solutions were collected and replaced with fresh solutions at 6 or 24 hour intervals for four days at which time the remaining tissue was collected and hydrolyzed.

Hydrolysis

One half milliliter of 6N HCl was added to the remaining tissue sample in a hydrolysis tube and flushed with nitrogen gas (N_2) for 30 seconds. One half milliliter of 12N HCl was added in a hydrolysis tube to the 0.5ml buffer or protease solutions collected at the various time points and each hydrolysis solution was flushed with nitrogen gas (N_2) for 30 seconds. All tubes were then capped and placed in an oven at 110°C for 24 hours or 150°C for 1 hour to hydrolyze. Hydrolyzed samples were transferred into 1.5ml micro-centrifuge tubes and 2ul to 40ul alliquots of the samples were pipetted into the wells of a 96 well Costar tissue culture plate. Each sample was assayed in replicates of four so that 20 samples could be assayed per plate. The plates were then placed into a heated vacuum chamber until the samples were dry. Forty microliters of QH_2O was added to each well to soluablize the dry residue before assay.

Hydroxyproline Assay

Reagents: 1.) 7% chloramine T in H_2O

2.) Citrate acetate buffer: 57g NaAcetate · H_2O
37.5g Na_3 citrate · H_2O
5.5g H_3 citrate · H_2O
385ml isopropanol
make up to 1L w/H_2O

3.) 20g DMBA in 30ml of 60% perchloric acid

4.) stock solution of cis-4-hydroxy-L-proline (100ug/ml)

Preparation of solutions:

Solution A: 1 volume of reagent 1 with 4 volumes of reagent 2

Solution B: 3 volumes of reagent 3 with 13 volumes of isoprop.

The first column of wells (1A-1H) of the 96 well Costar plate was left blank while the second column was left for the hydroxyproline standard. The hydroxyproline standard was set up by adding 1, 2, 3, 4, 5, 6, 8, and 10ul of hydroxyproline and 39, 38, 37, 36, 35, 34, 32, and 30ul of QH_2O into rows A-H respectively, in column 2. Into all other wells were pipetted the samples. After adding the 40ul of QH_2O as described in **Hydrolysis**, the procedure was as follows for each well: 1.) add 20ul isopropanol and mix; 2.) add 10ul solution A, mix, and let stand for 5 minutes; 3.) add 130ul solution B, mix, and cover with a plate sealer; 4.) heat in 60°C water bath (by floating) for 25 minutes; 5.) cool in tap water (by floating) for 5 minutes; 6.) read at 555nm in an EIA reader within 30 minutes. The standard showed a linear relationship between the ug of hydroxyproline and the absorbance read. Since collagens I, II, III, and IV, the major species in tissue, are 10% hydroxyproline by weight, ten times the ug quantity of hydroxyproline corresponded to the amount of collagen present. Plotting ug of collagen versus absorbance for the standard yielded a straight line. Thus 4 values of absorbance for each sample were averaged and through linear regression on a Hewlett Packard 22S calculator, the amount (in ug) of collagen in the sample was determined. Multiplying by the appropriate dilution factor, the amount of collagen in 1ml was determined. The total amount of collagen in the original button was determined by adding collagen values in supernatants at the various time points to the amount of collagen remaining in the tissue sample.

Results

Effects of Neutral Proteases on Corneal Collagen

The first series of investigations showed that various neutral proteases could degrade intrinsic collagen to a small degree. The 3 peptidase solution, consisting of leucine aminopeptidase, carboxypeptidase A, and carboxypeptidase B, was shown to degrade 2.4 - 2.8% (14.5 -19ug) of the total collagen in 3mm corneal punches taken from young and mature rabbit eyes (FIG. 2 & TABLE 1) after 4 days of incubation. This degradation increased over time, which can be seen by the amount of collagen soluablized over the 24 hour intervals (FIG.2.1 & TABLE 1.1). Those punches placed in buffer C solution as a control, did not show any evidence of degraded/solubilized collagen.

In order to determine whether the proteases or the substrate was limiting in the case of the 3 peptidase solution, the concentration of the proteases was increased 5 times resulting in the degradation of 1.5 - 2.0% (8 - 10ug) of the total collagen (FIG. 3 & TABLE 2). Again, the degradation of collagen by the 3 peptidase solution increased with time. (FIG. 3.1 & TABLE 2.1).

Exposure of 3mm corneal punches to the original 3 peptidase solution for 3 days after 24 hour exposure to trypsin, degraded 3.5 - 5.5% (21 -29.7ug) of the total collagen. Trypsin alone degraded 0.5 - 5.5% (3 -28.7ug) of the total collagen as well in 4 days. The buffer C solution also caused the soluablization of approximately 1% (7.5ug) of the mature corneal punch (FIG. 4 & TABLE 3). Trypsin caused solubilization of the greatest amount of collagen in the first 24 hours and after that the amount solubilized decreased and leveled off. The buffer C seemed to have an increased degradative effect over time on the mature corneal (FIG. 4.1 & TABLE 3.1).

After denaturing the corneal punches by boiling and then exposing them to trypsin and buffer C solution it was found that trypsin degraded 100% (200 - 280ug) of the collagen present in both young and mature corneal punches. One hundred percent (200ug) of the collagen in the young corneal punch solubilized into buffer C solution as well. In contrast, only 85% (219ug) of the collagen in the

mature corneal punch was solubilized (FIG. 5 & TABLE 4). In all cases except the mature corneal punch in buffer C, >90% of the collagen was liberated in the first 24 hours and decreased thereafter until 100% was degraded/solubilized. The mature corneal punch had very different kenetics, where only 60% of the collagen liberated was present in the first 24 hour supernatant and leveled off in the later 3 samples (FIG. 5.1 & TABLE 4.1).

Elastase degraded 1 - 3% (3.7 - 19ug) of the total collagen present in the young and mature rabbit corneas in 4 days. Cathepsin G was shown to degrade 1 - 5% (5 - 36ug) of the total collagen present in the corneal punches in 4 days. Again, the buffer C solution solubilized some collagen from the mature corneal punch, this time about 3.2% (19ug) (FIG. 6 & TABLE 5). There seemed to be no relationship between the amount of collagen degraded/solubilized with either of these proteases or buffer C (FIG. 6.1 & TABLE 5.1).

The neutral protease chymotrypsin was very effective degrading approximately 4 - 13% (19 - 56ug) of the total collagen present in the young and mature corneal punches in 4 days. Plasmin, while not at all effective against the mature corneal punch, degraded 3.5% (20ug) of the young in 4 days. Almost 4% (25ug) of the mature 3mm corneal punch was liberated in the buffer C solution (FIG. 7 & TABLE 6). Plasmin seemed to have an increased effect on degradation of collagen over time. Solubilization increased with time in the buffer C solution. Chymotrypsin did not show an increase in degradation over time (FIG. 7.1 & TABLE 6.1).

In order to determine shorter time kenetics on the various proteases, another study was done over 18 hours and a sample was taken at 6 hour time points. It was shown that the 3 peptidase solution was not effective in this shorter time period on either the mature or the young corneal punches. Cathepsin G likewise did not have any effect on the mature corneal punch. The rest of the proteases including elastase, plasmin, and trypsin had proportional effects in 18 hours as they did in 4 days. For instance elastase degraded 18.7 ug of collagen in the young cornea in 4 days and 3.2 ug in 18 hours. If carried on for another 78 hours, elastase theoretically would degrade 17.1 ug of collagen. These knetics are

fairly consistent and evident for the other proteases as well (FIG. 8 & TABLE 7). No linear increase in degradation with time existed with any of the proteases or buffer C as or collagen was degraded between 12 and 18 hours (FIG. 8.1 & TABLE 7.1).

<u>Solubilization of Corneal Collagen in Buffer C</u>

Several experiments were performed to determine if there was a regional aspect of the cornea which would effect the solubilization of its collagen into buffer C. Thus 3 regions of the cornea were defined and corneal punches from each region of both young and mature animals were denatured or not denatured and exposed to buffer C, receiving fresh solution at 6 hour intervals. Denatured young corneal punches liberated from 63 - 73% (504 - 718ug) of the total collagen present while native young corneal punches liberated only 1.3% (14ug) of the total collagen. Similarly, native mature corneal punches liberated only 1.1 - 3.5% (13 - 42ug) of the total collagen. But, the denatured mature corneal punches liberated 22 - 33% (225 - 335ug) of the total collagen, half of what was liberated by the young denatured corneal punches (FIGS. 9 & 10 and TABLES 8 & 9). Almost half of the total collagen liberated in both young and mature denatured corneal punches was in the supernatant in which the punches were boiled. Most of the remaining half was found in the supernatant collected at the first 6 hour sample collection. No other trend was noticed (FIGS. 9.1 & 10.1 and TABLES 8.1 & 9.1).

Solubilization of native collagen into buffer C in 24 hour increments yielded very different results from the previous procedure, as no collagen was found to have soluablized (FIG. 11 & TABLE 10).

To investigate the solubilization of collagen from a 2mm peripheral ring of a 6mm central corneal punch, demonstrated by Dr. Barbara Johnson-Wint, corneas were dissected in that manner. The 2mm peripheral rings were cut into superior, inferior, lateral and medial sections and incubated in buffer C. The lateral and medial sections in both young and mature corneal material consistantly had

some solubilization of collagen (1 to 6.6 ug) in 4 days (FIG. 12 & TABLE 11).

To determine whether or not the solubilization of the denatured corneal collagen was due (partially or wholly) to intrinsic proteases, a protease inhibitor cocktail was added to the buffer C solution in which the corneal punches were boiled. Denatured young corneal punches averaged a liberation of up to 47% (343ug) of their total collagen in 24 hours, while the replicates of these with inhibitor added, liberated only up to 39% (305ug) of their total collagen. Denatured mature corneal punches averaged a liberation of up to 31% (306ug) of the total collagen in 24 hours. The replicates of these with inhibitor added, liberated up to 27% (250ug) of their total collagen (FIG. 13 & TABLE 12).

Determination of the Total Amount of Collagen

To determine the total amount of collagen present in a 3mm punch from the three defined regions, 3 young and 3 mature rabbit eyes were dissected and 3mm punches were taken from the edge, center, and intermediate zone of the cornea (FIG. 1). Average ug quantities of collagen were determined for the regions of each young animal (FIG. 14 & TABLE 13) and each mature animal (FIG. 15 &TABLE 14). Young corneal punches from the center averaged, 1,771ug; the edge, 1,659ug; and the intermediate zone 1,652ug. Mature corneal punches from the center averaged 1,986ug; the edge, 1,947ug; and the intermediate zone 2,058ug (FIG. 16 & TABLE 15). These values did not differ enough to determine that the defined corneal regions contain different amounts of collagen. But, it can be said that the mature rabbit cornea contains more collagen than the young.

Degradation of Collagen After Denaturation

To determine fragment size of collagen after it was denatured by boiling, central punches from 3 mature and 3 young animals were taken and boiled. A 0.1 ml alliquot was taken from the boiled sample and the rest was placed in a 3,500 molecular weight cut-off dialysis bag and dialyzed against H_2O. After dialysis for 24 hours

samples were removed and hydrolyzed. Hydroxyproline assay revealed that a similiar amount of collagen was present in the sample before (540 ug) and after (535 ug) dialysis for the young corneal punches. In the samples for the mature corneal punches it seems that some collagen had dialized out indicating some fragment sizes less than 3,500 molecular weight (FIG. 17 & TABLE 16).

Discussion

The investigations of long-term protease action on interstitial collagen proved to be significant, as most had some degradative effect. The 3 peptidase solution for example, degraded from 2.4% to 2.8% of the total collagen in corneal punches in 4 days. Hence, it would take approximately 167 days and 147 days to completely degrade the young and mature corneal punches respectively. It was also shown that an increased concentration of the 3 peptidase solution had no different effects on collagen degradation as only 1.5% to 2% total collagen was degraded. This proved that the substrate (collagen) was limiting, not the enzymes. There must have been only a certain number of ends the exopeptidases would work on in the tissue punch. More of these peptide ends may have become available as the peptidases began working, because there was a slight increase in degradation rate over time.

Using the sequenced calf skin collagen molecule shown by Piez and Reddi, the number of possible cleavage sites for each exopeptidase on a denatured collagen molecule was determined. Due to the extreme specificities of leucine aminopeptidase and carboxypeptidase B, they have 26 and 77 cleavage sites respectively. This of course, is only possible if other residues are cleaved off (by other proteases) leaving the residues that leucine aminopeptidase can cleave as N-terminal. By the same token, it would be necessary for other proteases to cleave other residues to expose the ones carboxypeptidase B is specific for as C-terminal ends. Since carboxypeptidase A can cleave any C-terminal residue except proline, histidine, arginine, or lysine, it has 1264 cleavage sites on the denatured molecule, provided the residues were presented as C-terminal. Obviously, if these are exopeptidases fragments sizes would be one to few residues in length.

The investigation involving exposure of collagen to trypsin and then the 3 peptidase showed 3.6 - 5.4% degradation of total collagen. This is more evidence of an increase in peptide ends available to the exopeptidases as trypsin can cleave the middle regions of denatured collagen. Trypsin itself was also effective for these

reasons, degrading 0.4 - 5.4 % of the total collagen. Hence, trypsin would totally degrade the young and mature corneal punches it was degrading in 74 and 1000 days (2.5 years!) respectively. The mature corneal punch is obviously much more resistant to the trypsin, probably due to crosslinkages. Specific for carboxyl ends of arginine and lysine, it was determined that trypsin has 77 possible cleavage sites in denatured calf skin collagen. Thus trypsin would create fragments of relatively few peptidases long (approximately 18 residues).

This investigation was the first involving an inconsistent solubilization of a variable amount of collagen in neutral buffer. It was postulated that since some of these punches were stored in buffer C for extended periods of time (up to 2 weeks) that non-crosslinked collagen could solubilize out. The solubilization of a variable amount of collagen from mature corneal punches in neutral buffer was again seen in the following 3 experiments. Only in one experiment (the last of these 3 experiments) did any collagen solubilize from the young corneal punches in buffer C. This punch was taken from the intermediate zone of the cornea. Decreases in values for total amounts of collagen were also seen - probably due to a solubilization while storing. To test this hypothesis, a later experiment was performed where corneal punches were placed in buffer C immediately after dissection. Samples were collected every 24 hours for four days. No collagen was found to have solubilized in any of the 24 hour increments, indicating our hypothesis may have been correct.

Denaturing the collagen to gelatin by boiling resulted in its solubilization from the tissue. The mature animal showed solubilization of 85% of its total collagen and the young 100%, in buffer C. This would indicate a difference in the collagen regarding age. Possibly the mature animal's cornea is more extensively crosslinked and this 15% was denatured but couldn't get out of the tissue. This was seen again in a later experiment where heat denaturation resulted in 63% - 73% of the total collagen solubilizing in young corneal punches. Again further inconsistencies arose in solubilization of native collagen in buffer C even though these

punches were used immediately. When the denatured collagen was exposed to trypsin however, 100% of the collagen was solubilized in both young and mature punches, as trypsin could cut up any remaining crosslinked collagen and allow it to come out into solution.

The solubilization of denatured collagen could have been due (partially or wholly) to intrinsic proteases present in the corneal punch. To test this hypothesis a protease inhibitor cocktail was added to the buffer C solution in which the corneal punches were boiled. The cocktail was designed to inhibit all known classes of proteases, so that if collagen solubilization was at all due to protease action, a decrease in collagen solubilized would indicate this. An 8% decrease in total collagen solubilized in young corneal punches was shown as well as a 4% decrease in mature corneal punches. This would indicate that intrinsic protease action is partially responsible for solubilization of denatured collagen. This is probably best explained by increased vulnerability of the collagen to the proteases after denaturation. Further study could include identifying the exact proteases involved by selective inhibition of the proteases.

The neutral proteases, elastase, cathepsin G, plasmin and chymotrypsin also had a degradative effect on collagen. Chymotrypsin was the most effective, degrading 4 - 13% of the total collagen. Hence, if it had kept working, chymotrypsin would have completely degraded it's young and mature corneal punches in 105 and 40 days respectively. Chymotrypsin cleaves only at carboxyl ends of aromatic amino acids and thus has only 12 specific cleavage sites in the calf skin collagen molecule. This would lead to rather long peptide chain fragments of approximately 113 residues. Plasmin, having the same specificity as trypsin, strangely was the least effective as it did not effect the mature corneal punch. However, it would have totally degraded the young corneal punch it was working on in approximately 118 days, as it had degraded 3.4% of the collagen in 4 days. This may have been due to abnormally extensive crosslinking or other characteristics possessed by that particular individual rabbit. This also may have been due to the protease itself, effecting its specificity.

Cathepsin G would have degraded it's young and mature corneal punches in 455 and 80 days respectively, as it degraded 0.88% and 5% in 4 days. Conversely, elastase would have degraded it's young and mature corneal punches in 138 and 455 days respectively, as it degraded 2.9% and 0.88% in 4 days. It seems that elastase had the same effects of opposite intensity on the young and mature corneal punches. Elastase is specific for cleaving peptides adjacent to neutral residues and thus has 1217 specific cleavage sites in the calf skin molecule. Fragments created then, would be extremely small and probably single amino acid residues.

Preliminary studies by Dr. Barbara Johnson-Wint show a marked and consistent increased solubilization of collagen in buffer C by a 2mm peripheral ring compared to a 6mm central corneal punch. A final experiment involving this approach showed a consistent solubilization from the lateral and medial sections of a 2mm peripheral ring of a 8mm central corneal punch. This could be as mentioned, due to blinking. The lateral and medial sections would contain the horizontal axis of foreign particles and tear growth factors created by blinking. This would create some regional physiological differences on the surface of the eye leading to differences in collagen solubility and turnover in this region, possibly due to fewer crosslinks. For example, it was previously shown that a significant net increase of total collagen synthesis in the peripheral area of grafted rabbit corneas, was greater than that in the central area (Lass, 1987). We were not able to determine a pronounced difference in the solubilization of the edge, center or intermediate zone of the cornea. This was probably due to the dissimilar approaches of the two studies. The corneal punches taken here are more random than those in the study by Dr. Johnson-Wint. It is therefore possible that the zone exhibiting this consistent increased collagen solubility is only sampled infrequently in my study.

To determine whether or not the total amount of collagen was different in the 3 defined regions of the cornea, 3mm punches were taken from the edge, center, and intermediate zone of the cornea. It was shown that no regional differences existed, but that age was a

factor. The mature corneal punches contained 15% more collagen than the young corneal punches.

Further studies related to the topics discussed include the investigation of effects of other proteases including cysteine proteases like cathepsin B, which are known to degrade collagens. (Emonard 1990). The previously mentioned selective inhibitor study to determine which intrinsic proteases actively degrade denatured collagen is promising as well. Also, by electrophoretic gels, molecular weight sizes of the solubilized collagen could be determined.

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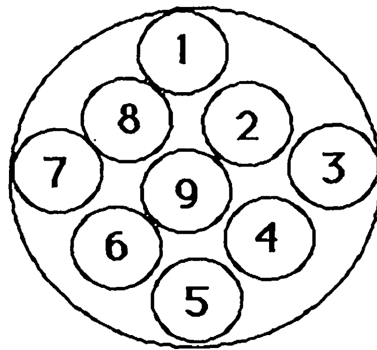

FIG. 1. Schematic representation of the 3mm punches taken from the rabbit cornea. 1, 3, 5, and 7 = edge of cornea; 2, 4, 6 = intermediate zone of cornea; 8 and 9 = center of cornea.

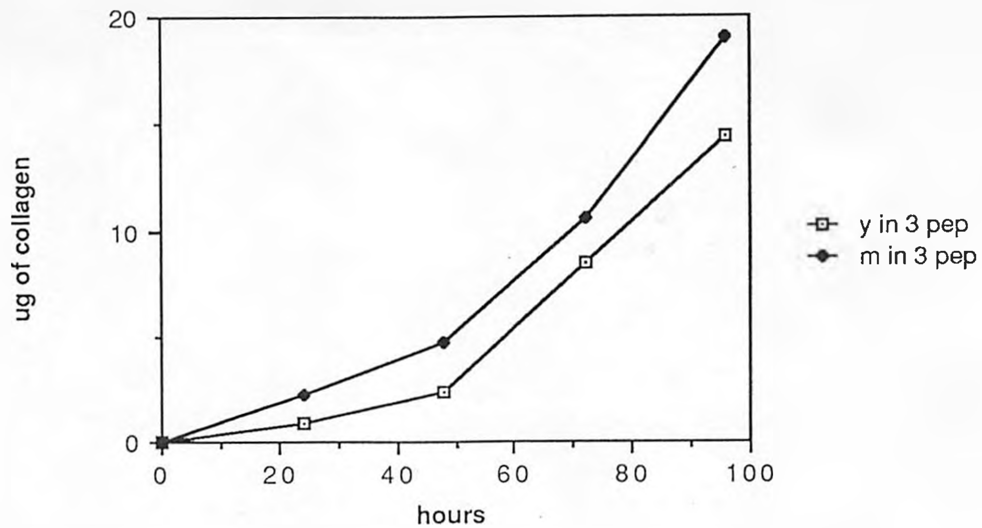

FIG. 2. Total degradation of collagen in corneal punches in 3 peptidase solution at 4 days. Open square = young corneal punches in 3 peptidase solution; closed diamond = mature corneal punches in 3 peptidase solution. Corneal punches were placed in buffer C as a control, zero values were not plotted but can be seen in TABLE 1.

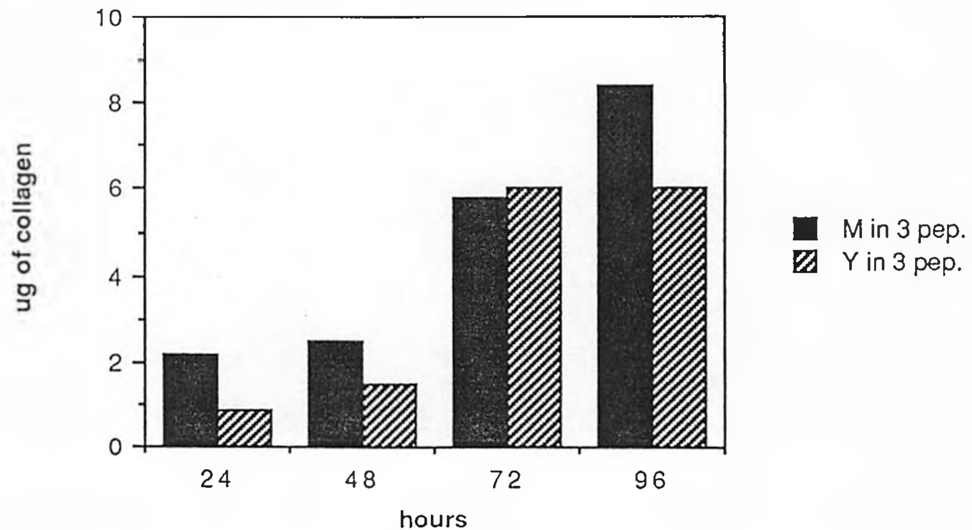

FIG. 2.1. Daily degradation of collagen in corneal punches in 3 peptidase solution in 4 days. Shaded bars = mature corneal punches in 3 peptidase solution; striped bars = young corneal punches in 3 peptidase solution. Corneal punches were placed in buffer C as a control, zero values can be seen in TABLE 1.1.

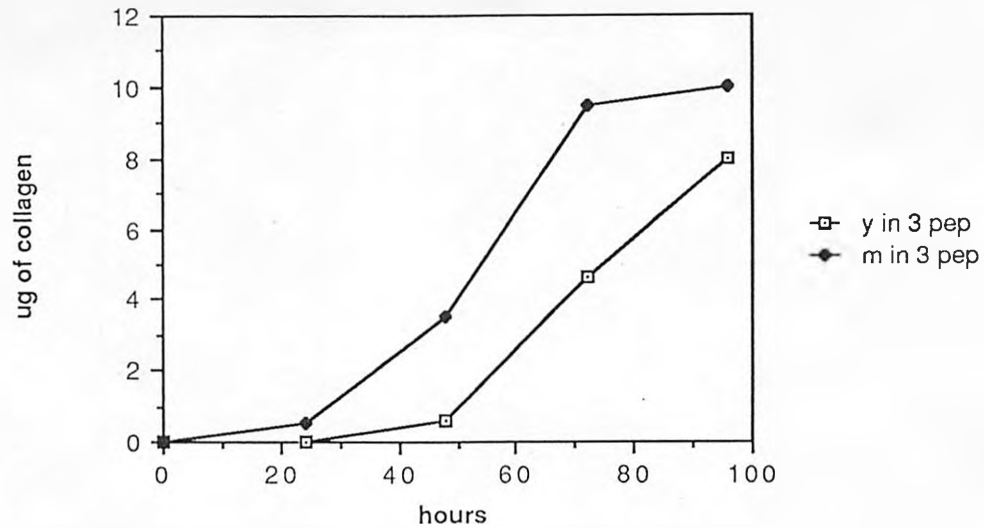

FIG. 3. Total degradation of collagen in corneal punches in 3 peptidase solution X 5 at 4 days. Open square = young corneal punches in 3 peptidase solution X 5; closed diamond = mature corneal punches in 3 peptidase solution X 5. Corneal punches were placed in buffer C as a control, zero values were not plotted but can be seen in TABLE 2.

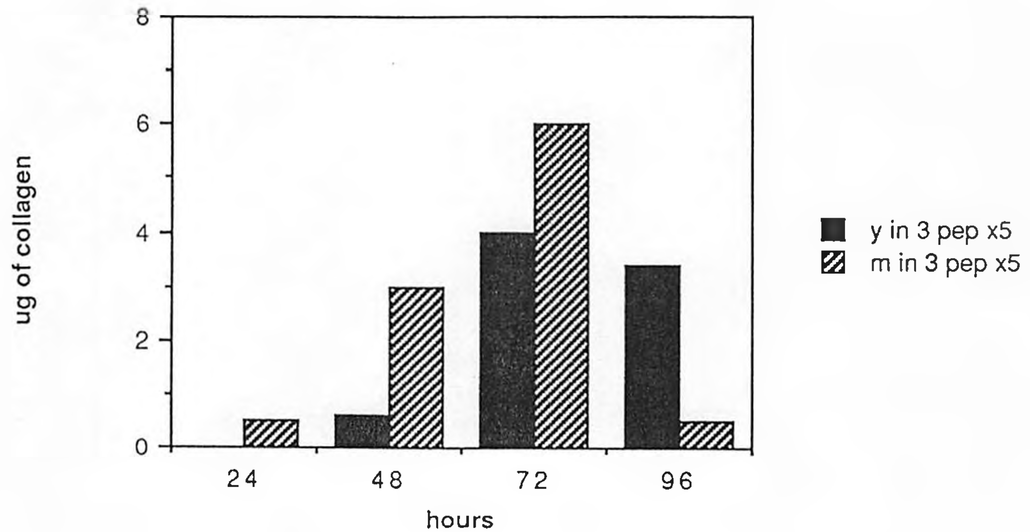

FIG. 3.1. Daily degradation of collagen in corneal punches in 3 peptidase solution X 5 in 4 days. Shaded bars = young corneal punches in 3 peptidase solution X 5; striped bars = mature corneal punches in 3 peptidase solution X 5. Corneal punches were placed in buffer C as a control, zero values can be seen in TABLE 2.1.

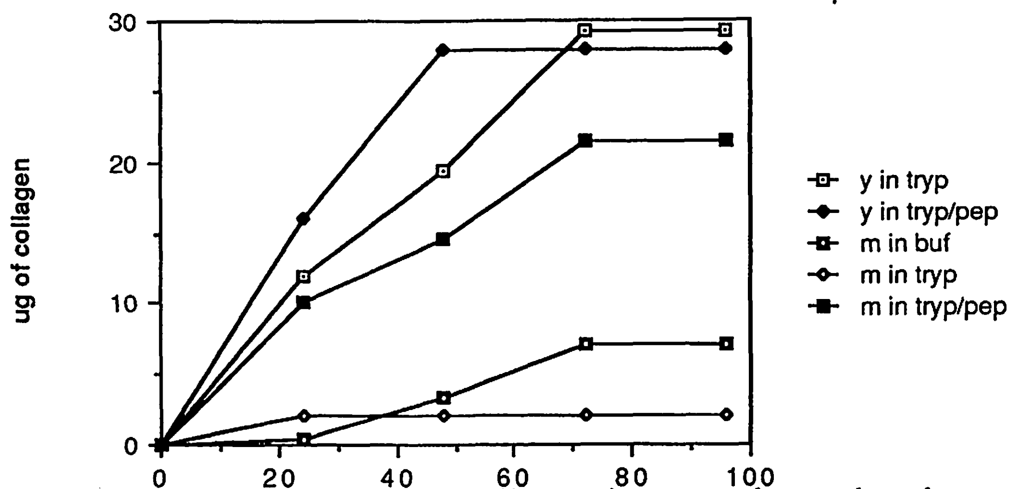

FIG. 4. Total degradation of collagen in corneal punches in trypsin for 4 days and in trypsin for 24 hours followed by 3 peptidase solution for 72 hours. Open square = young corneal punches in trypsin; closed diamond = young corneal punches in trypsin followed by 3 peptidase solution; closed square with open dot = mature corneal punches in buffer C; open diamond = mature corneal punches in trypsin; closed square = mature corneal punches in trypsin followed by 3 peptidase solution. Corneal punches were placed in buffer C as a control, zero values were not plotted but can be seen in TABLE 3.

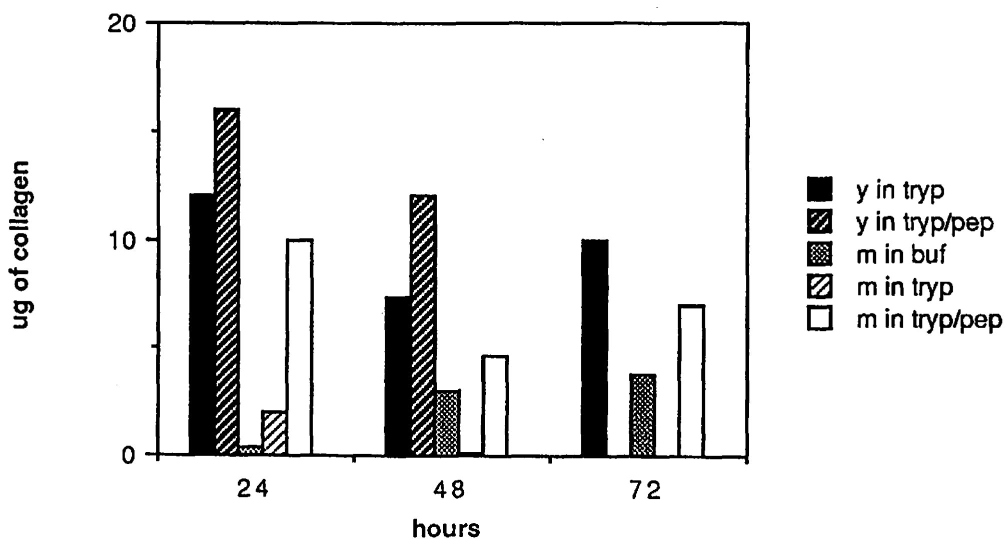

FIG. 4.1. Daily degradation of collagen in corneal punches in trypsin for 4 days and in trypsin for 24 hours, followed by 3 peptidase solution for 72 hours. Shaded bars = young corneal punches in trypsin; wide striped bars = young corneal punches in trypsin followed by 3 peptidase solution; criss-crossed bars = mature corneal punches in buffer C solution; thin striped bars = mature corneal punches in trypsin; open bars = mature corneal punches in trypsin followed by 3 peptidase solution. Corneal punches were placed in buffer C as a control, zero values can be seen in TABLE 3.1.

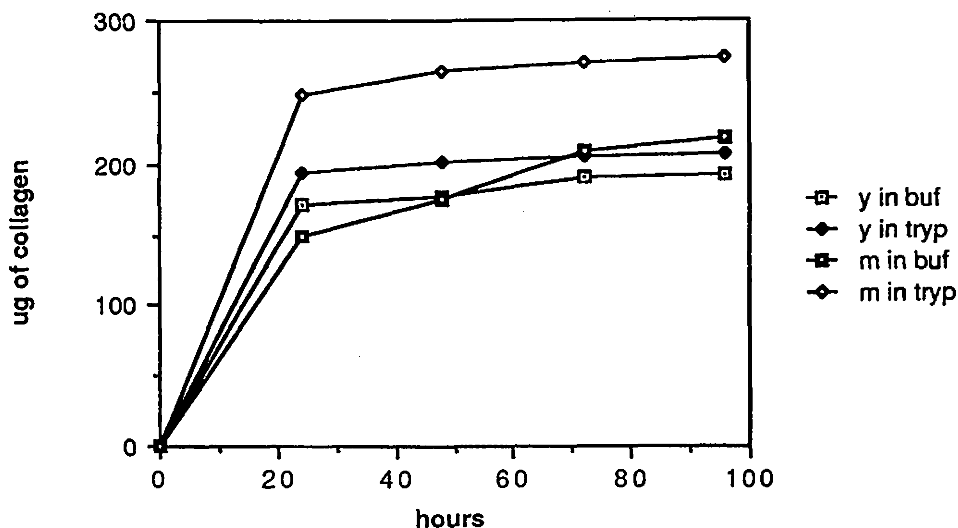

FIG. 5. Total solubilization or degradation of collagen in corneal punches in trypsin and buffer C after denaturation by boiling at 4 days. Open square = young corneal punches in buffer C; closed diamond = young corneal punches in trypsin; closed square = mature corneal punches in buffer C; open diamond = mature corneal punches in trypsin.

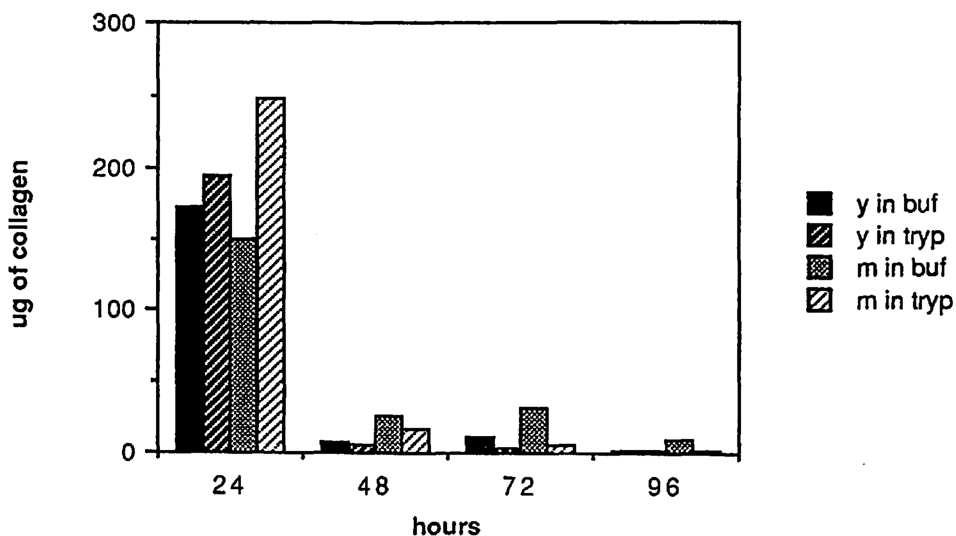

FIG. 5.1. Daily solubilization or degradation of collagen in corneal punches in trypsin and buffer C after denaturation by boiling in 4 days. Shaded bars = young corneal punches in buffer C; wide striped bars = young corneal punches in trypsin; criss-crossed bars = mature corneal punches in buffer C; thin striped bars = mature corneal punches in trypsin.

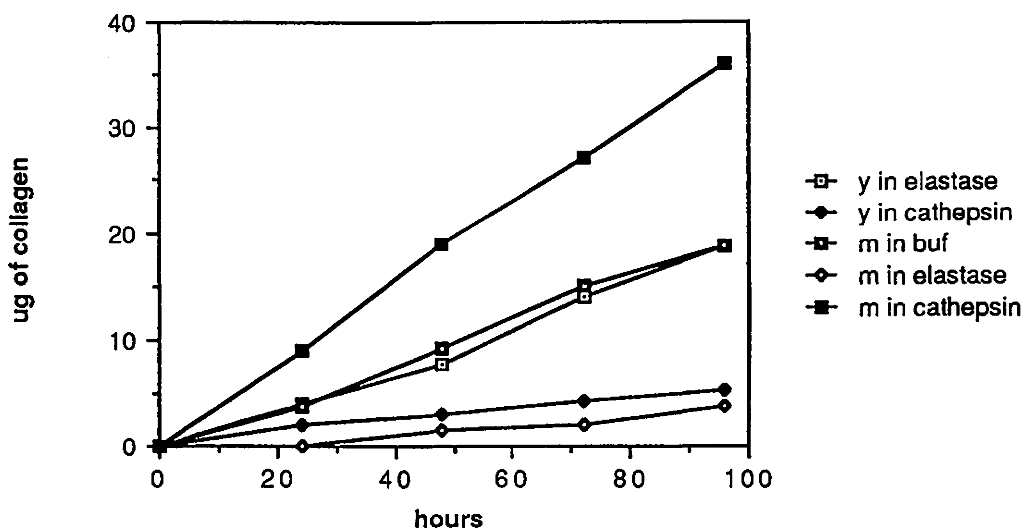

FIG. 6. Total degradation of collagen in corneal punches in elastase and cathepsin G at 4 days, Open square = young corneal punches in elastase; closed diamond = young corneal punches in cathepsin G; closed square with open dot = mature corneal punches in buffer C; open diamond = mature corneal punches in elastase; closed square = mature corneal punches in cathepsin G. Corneal punches were placed in buffer C as a control, zero values were not plotted but can be seen in TABLE 5.

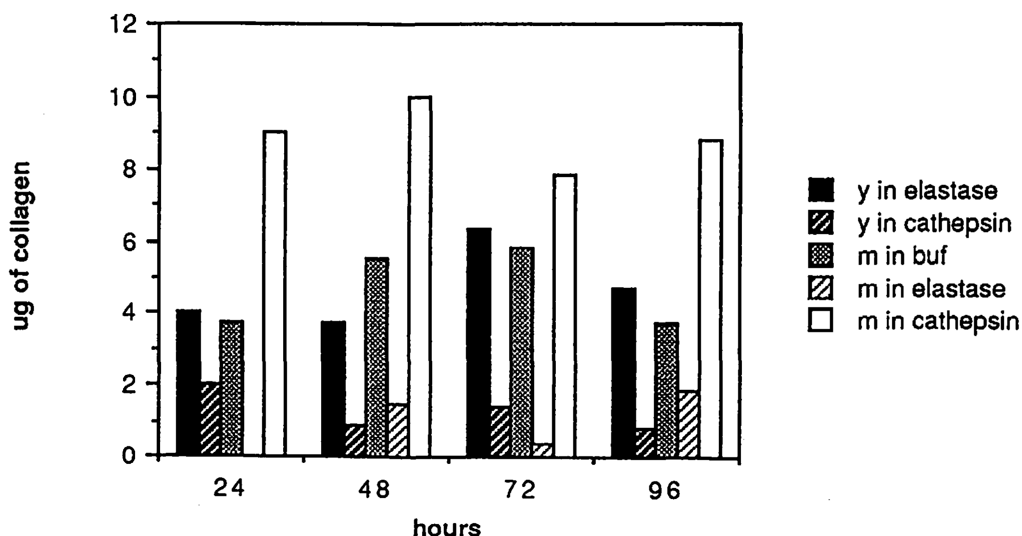

FIG. 6.1. Daily degradation of collagen in corneal punches in elastase and cathepsin G in 4 days. Shaded bars = young corneal punches in elastase; wide striped bars = young corneal punches in cathepsin G; criss-crossed bars = mature corneal punches in buffer C; thin striped bars = mature corneal punches in elastase; open bars = mature corneal punches in cathepsin G. Corneal punches were placed in buffer C as a control, zero values can be seen in TABLE 5.1.

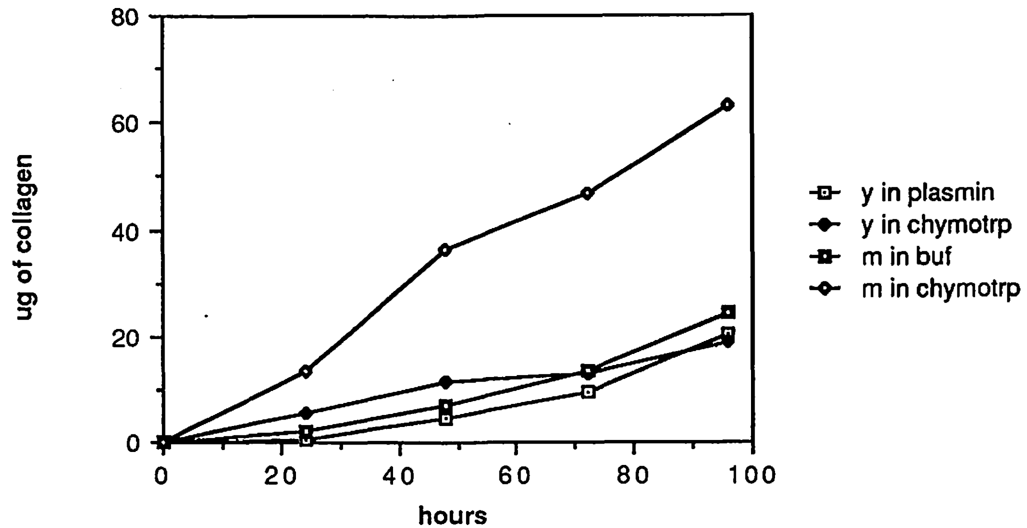

FIG. 7. Total degradation of collagen in corneal punches in plasmin and chymotrypsin at 4 days. Open square = young corneal punches in plasmin; closed diamond = young corneal punches in chymotrypsin; closed square = mature corneal punches in buffer C; open diamond = mature corneal punches in chymotrypsin. Corneal punches were placed in buffer C as a control, zero values were not plotted but can be seen in TABLE 6.

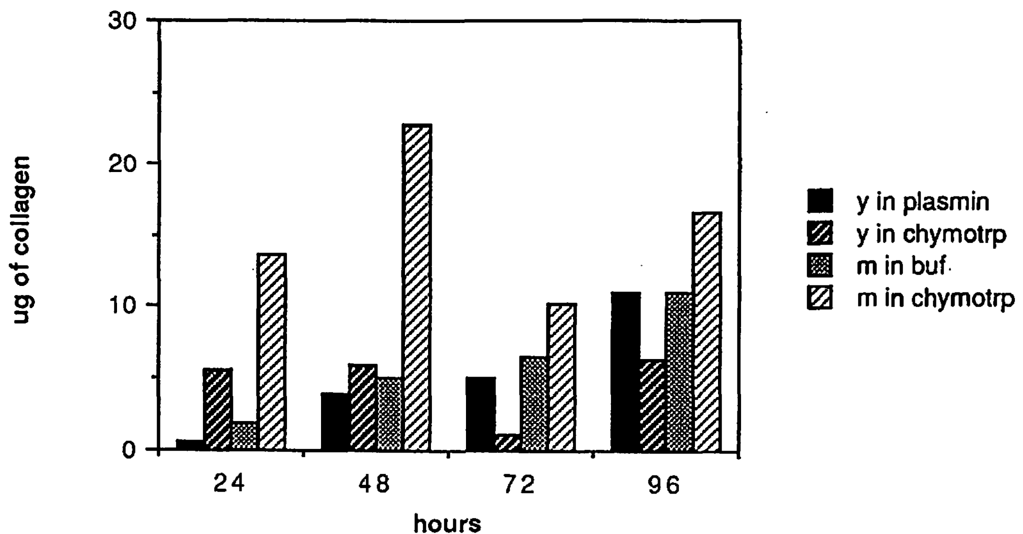

FIG. 7.1. Daily degradation of collagen in corneal punches in plasmin and chymotrypsin in 4 days. Shaded bars = young corneal punches in plasmin; wide striped bars = young corneal punches in chymotrypsin; criss-crossed bars = mature corneal punches in buffer C; thin striped bars = mature corneal punches in chymotrypsin. Corneal punches were placed in buffer C as a control, zero values can be seen in TABLE 6.1.

TOTAL DEGRADATION IN NEUTRAL PROTEASES

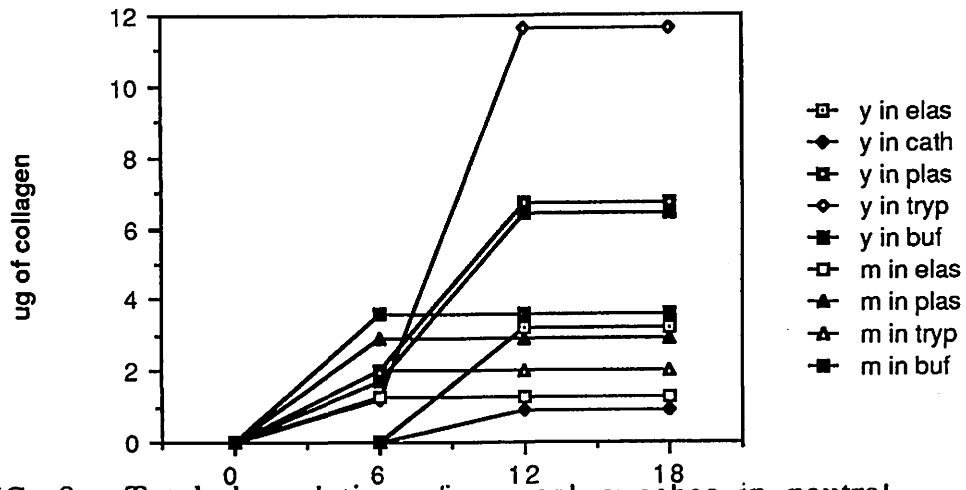

FIG. 8. Total degradation of corneal punches in neutral proteases. Open square with a closed dot = young corneal punches in elastase; closed diamond = young corneal punches in cathepsin G; closed square with an open dot = young corneal punches in plasmin; open diamond = young corneal punches in trypsin; closed square = young corneal punches in buffer C; open square = mature corneal punches in elastase; closed triangle = mature corneal punches in plasmin; open triangle = mature corneal punches in trypsin; closed square with open diamond = mature corneal punches in buffer C. Zero values were not plotted but can be seen in TABLE 7.

HOURLY DEGRADATION IN NEUTRAL PROTEASES

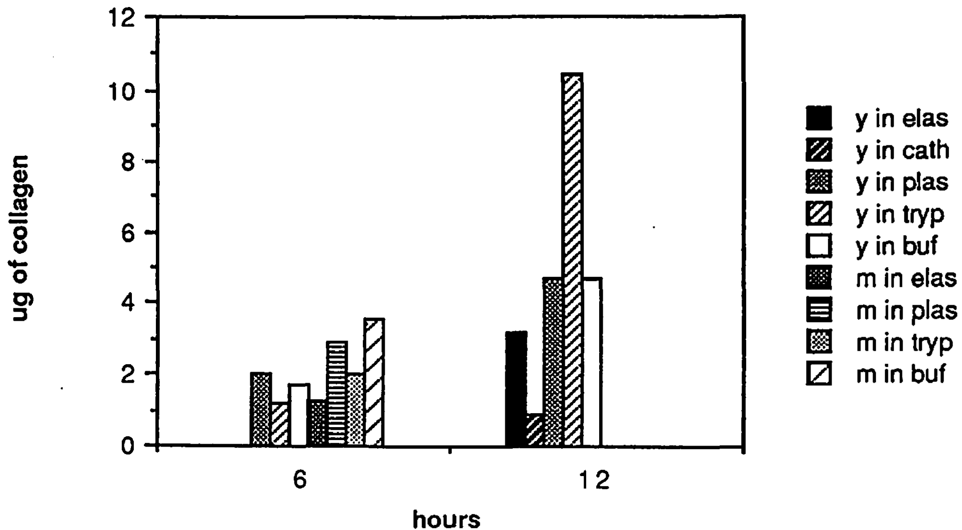

FIG. 8.1. Hourly degradation of corneal punches in neutral proteases. Shaded bars = young corneal punches in elastase; wide striped bars = young corneal punches in cathepsin G; criss-crossed bars = young corneal punches in plasmin; thin striped bars = young corneal punches in trypsin; open bars = young corneal punches in buffer C; polka-dot bars = mature corneal punches in elastase; horizontal striped bars = mature corneal punches in plasmin; dashed bars = mature corneal punches in trypsin; very thin striped bars = mature corneal punches in buffer C. Zero values can be seen in TABLE 7.1.

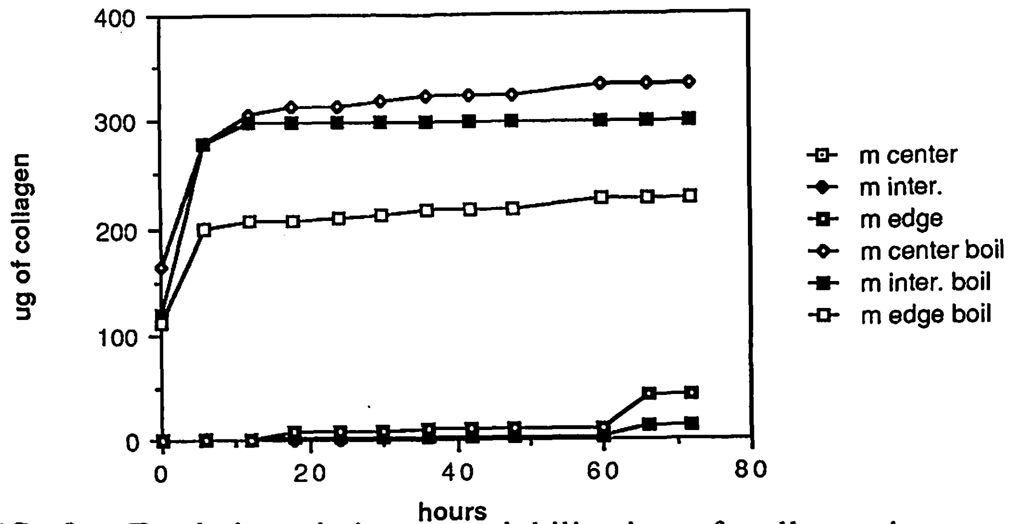

FIG. 9. Total degradation or solubilization of collagen in mature corneal punches without, and after denaturation by boiling at 3 days. Open square with a closed dot = center mature corneal punches in buffer C; closed diamond = intermediate mature corneal punches in buffer C; closed square with open dot = edge mature corneal punches in buffer C; open diamond = boiled center mature corneal punches in buffer C; closed square = boiled intermediate mature corneal punches in buffer C; open square = boiled edge mature corneal punches in buffer C.

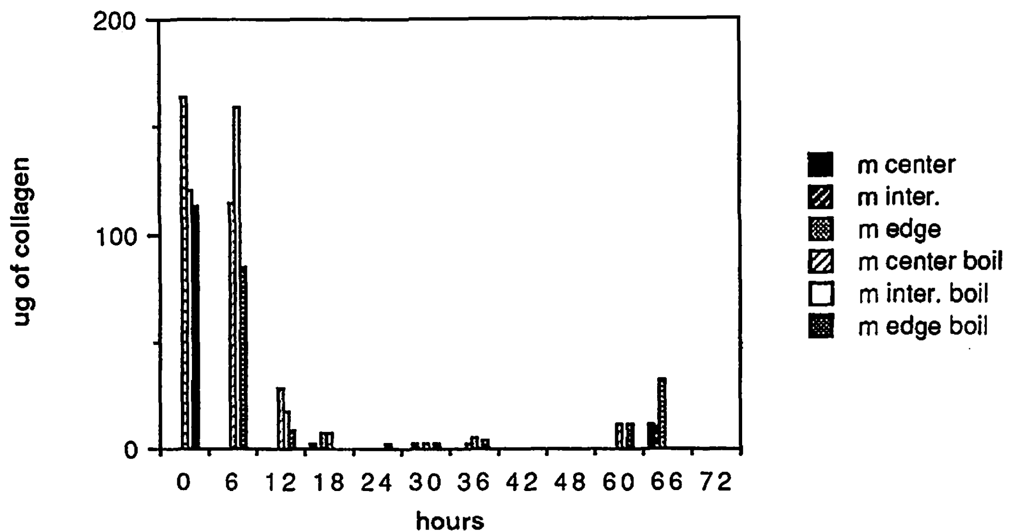

FIG. 9.1. Hourly degradation or solubilization of collagen in mature corneal punches without, and after denaturation by boiling every 6 hours. Shaded bars = center mature corneal punches in buffer C; wide striped bars = intermediate mature corneal punches in buffer C; criss-crossed bars = edge mature corneal punches in buffer C; thin striped bars = boiled center mature corneal punches in buffer C; open bars = boiled intermediate mature corneal punches in buffer C; shaded bars with open holes = boiled edge mature corneal punches in buffer C.

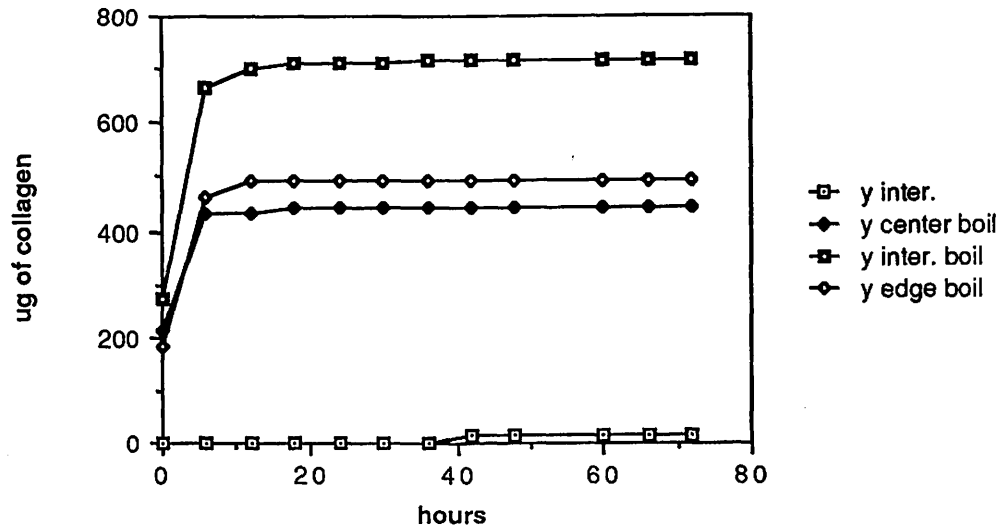

FIG. 10. Total degradation or solubilization of collagen in young corneal punches without, and after denaturation by boiling at 3 days. Open square = intermediate young corneal punches in buffer C; closed diamond = boiled center young corneal punches in buffer C; closed square = boiled intermediate young corneal punches in buffer C; open diamond = boiled edge young corneal punches in buffer C. Zero values were not plotted, but can be seen in TABLE 9.

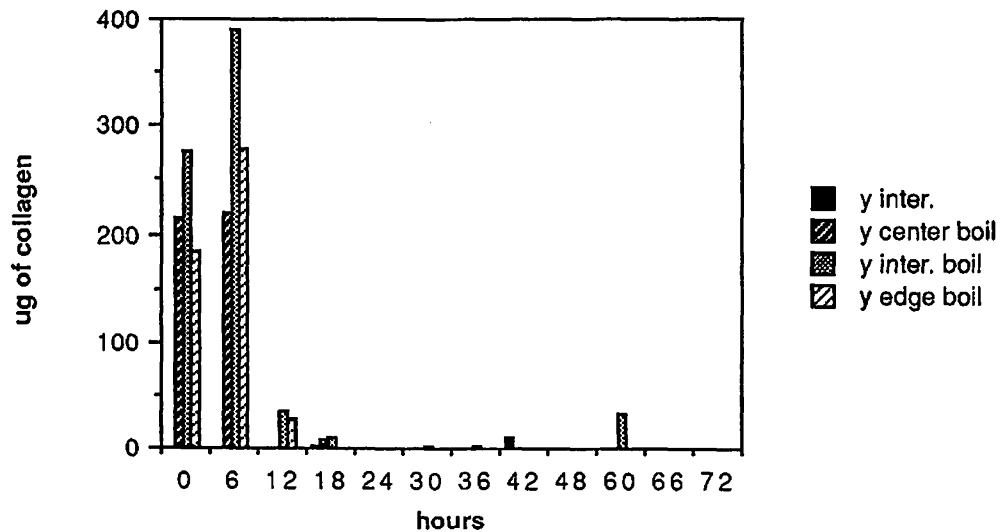

FIG. 10.1. Hourly degradation or solubilization of collagen in young corneal punches without, and after denaturation by boiling every 6 hours. Shaded bars = intermediate young corneal punches in buffer C; wide striped bars = boiled center young corneal punches in buffer C; criss-crossed bars = boiled intermediate young corneal punches in buffer C; thin striped bars = boiled edge young corneal punches in buffer C. Zero values can be seen in TABLE 9.1.

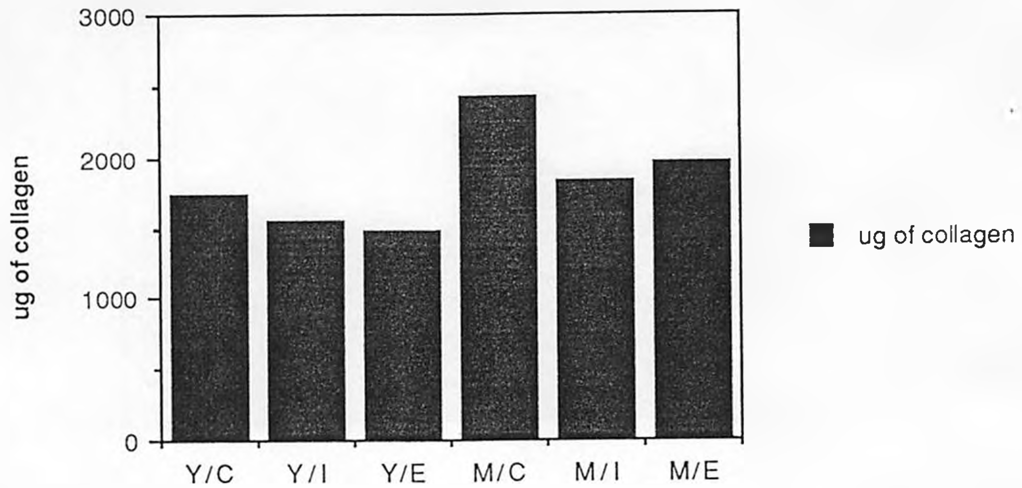

FIG. 11. Total collagen in 3mm corneal punches from which no collagen solubilized from buffer C in 4 days. Y/C = young center corneal punch; Y/1 = young intermediate in corneal punch; Y/E = young edge corneal punch; M/C = mature center corneal punch; M/E = mature edge corneal punch; shaded bars = total ug of collagen present in corneal punch.

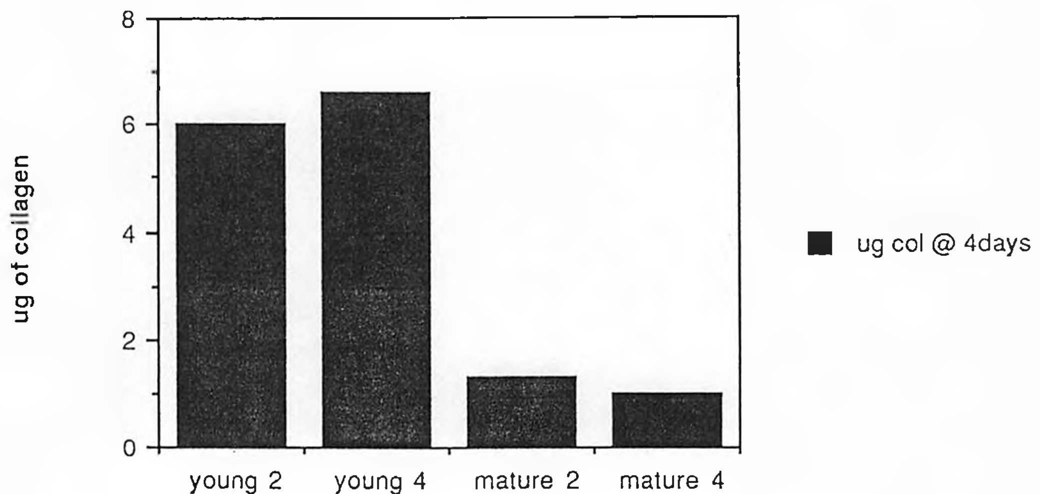

FIG. 12. Solubilization of a 2mm peripheral ring of a 6mm central corneal punch in 4 days. young 2 = medial section of a 2mm peripheral ring of young cornea; young 4 = lateral section of 2mm peripheral ring of young cornea; mature 2 = medial section of 2mm peripheral ring of mature cornea; mature 4 = lateral section of 2mm peripheral ring of mature cornea; shaded bars = ug of collagen solubilized in 4 days. Zero values were not plotted but can be seen in TABLE 11.

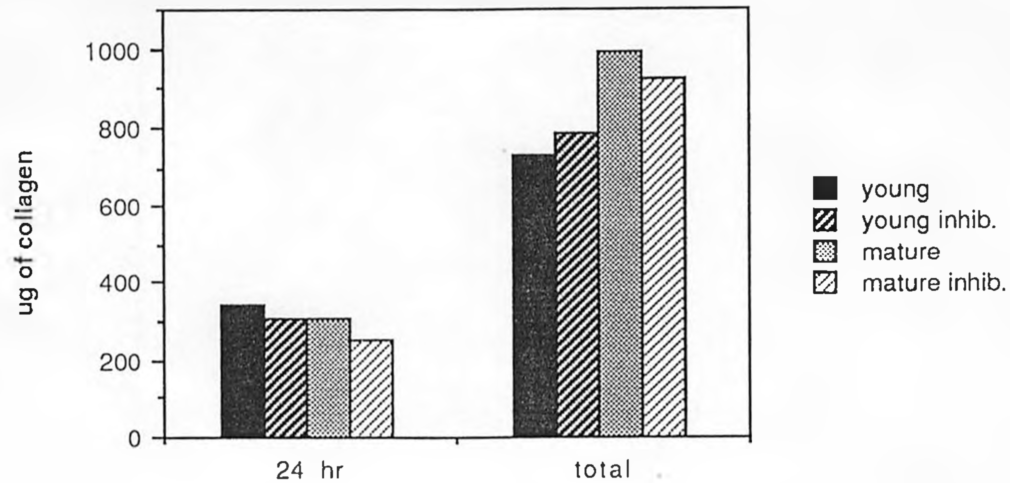

FIG. 13. Total solubilization of collagen in corneal punches after boiling with inhibitor cocktail versus without at 24 hours. Shaded bars = boiled young corneal punches in buffer C; wide striped bars = boiled young corneal punches in inhibitor cocktail; criss-crossed bars = boiled mature corneal punches in buffer C; thin striped bars = boiled mature corneal punches in inhibitor cocktail. 24hr = amount of collagen solubilized at 24 hours; total = total amount of collagen present.

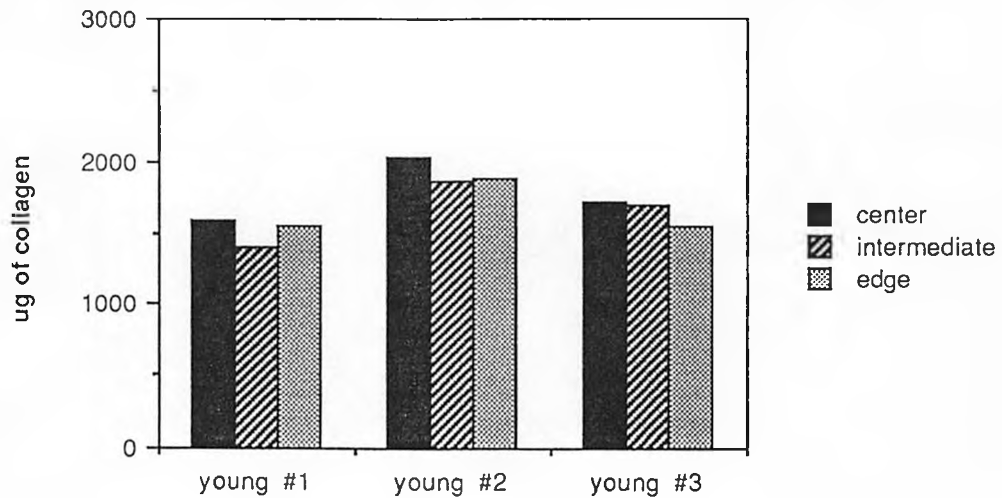

FIG. 14. Total collagen in young corneal punches. Shaded bars= center young corneal punches; wide striped bars = intermediate young corneal punches; criss-crossed bars = edge young corneal punches; young #1 = young animal eye #1; young #2 = young animal eye #2; young #3 = young animal eye #3.

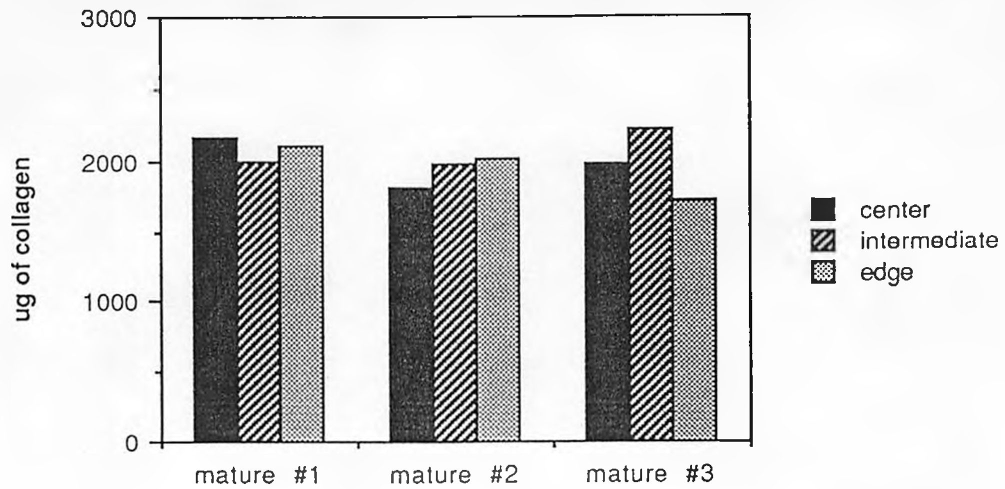

FIG. 15. Total collagen in mature corneal punches. Shaded bars = center mature corneal punches; wide striped bars = intermediate mature corneal punches; criss-crossed bars = edge mature corneal punches; mature #1 = mature animal eye #1; mature #2 = mature animal eye #2; mature #3 = mature animal eye #3.

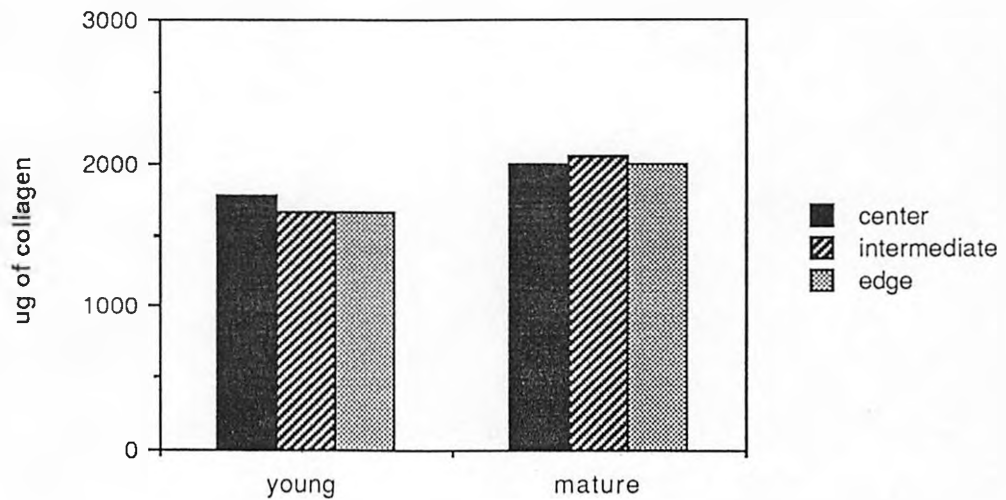

FIG. 16. Total collagen in corneal punches - a comparison between young and mature. Shaded bars = young and mature center corneal punches; wide striped bars = young and mature intermediate corneal punches; criss-crossed bar = young and mature edge corneal punches; young = young corneal punches; mature = mature corneal punches.

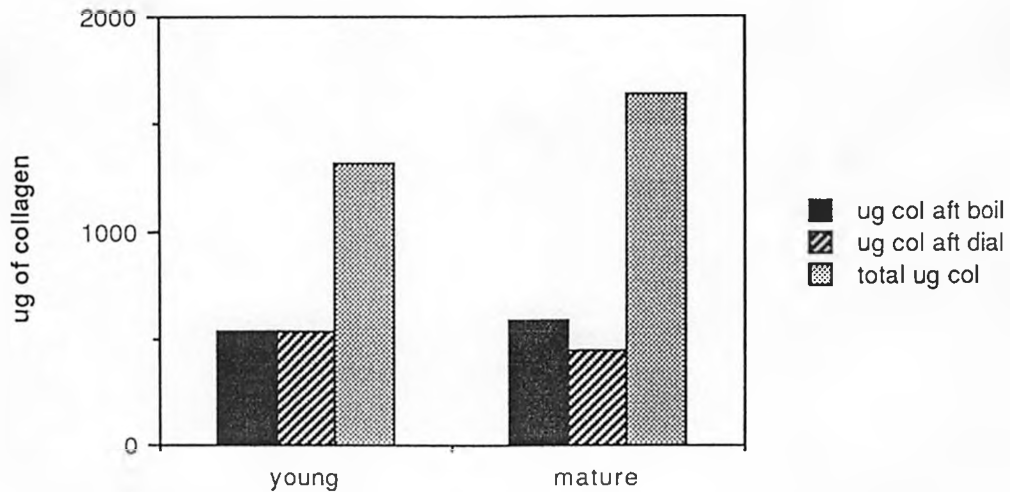

FIG. 17. Total collagen before and after dialysis of boiled young and mature corneal punches. Shaded bars = ug of collagen present in sample after boiling corneal punches; striped bars = ug of collagen present in sample after dialysis of boiled corneal punches; criss-crossed bars = total ug of collagen present in corneal punches; young = young center corneal punches; mature = mature center corneal punches.

TABLE 1: TOTAL DEGRADATION IN 3 PEPTIDASE SOLUTION

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr	total %
young buf	422	0	0	0	0	0
y in 3 pep	606	.875	2.4	8.4	14.4	2.4
mat buf	674	0	0	0	0	0
mat 3 pep	685	2.2	4.7	10.5	19	2.8

TABLE 1.1: DAILY DEGRADATION IN 3 PEPTIDASE SOLUTION

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr
young buf	422	0	0	0	0
y in 3 pep	606	.875	1.5	6	6
mat buf	674	0	0	0	0
mat 3 pep	685	2.2	2.5	5.8	8.4

TABLE 1 & 1.1. young buf = young corneal punches in buffer C; y in 3 pep = young corneal punches in 3 peptidase solution; mat buf = mature corneal punches in buffer C; mat 3 pep = mature corneal punches in 3 peptidase solution; tot ug col = total ug of collagen present in corneal punch; ug @ 24, 48, 72, 96 hr = ug of collagen degraded by that time (TABLE 1) or at that time (TABLE 1.1); total % = percentage of the total collagen degraded in 4 days.

TABLE 2: TOTAL DEGRADATION IN 3 PEPTIDASE SOLUTION X5

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr	total %
young buf	566	0	0	0	0	0
y in 3 pep	568	0	.6	4.6	8	1.4
mat buf	464	0	0	0	0	0
mat 3 pep	487	.5	3.5	9.5	10	2

TABLE 2.1: DAILY DEGRADATION IN 3 PEPTIDASE SOLUTION X5

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr
young buf	566	0	0	0	0
y in 3 pep	568	0	.6	4	3.4
mat buf	464	0	0	0	0
mat 3 pep	487	.5	3	6	.5

TABLE 2 & 2.1. young buf = young corneal punches in buffer C; y in 3 pep = young corneal punches in 3 peptidase solution times 5; mat buf = mature corneal punches in buffer C; mat 3 pep = mature

corneal punches in 3 peptidase solution times 5; tot ug col = total ug of collagen present in corneal punch; ug @ 24, 48, 72, 96hr = ug of collagen degraded by that time (TABLE 2) or at that time (TABLE 2.1); total % = percentage of total collagen degraded in 4 days.

TABLE 3: TOTAL DEGRADATION IN TRYPSIN & TRYPSIN/3 PEP. SOL.

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr	total %
young buf	472	0	0	0	0	0
y tryp.	540	12	19.3	29.3	29.3	5.4
y trp/pep	521	16	28	28	28	5.4
mat buf	647	.4	3.4	7.1	7.1	1.2
mat tryp	537	2	2.1	2.1	2.1	.4
mtrp/pep	601	10	14.6	21.5	21.5	3.6

TABLE 3.1: DAILY DEGRADATION IN TRYPSIN & TRYPSIN/3 PEP. SOL.

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr
young buf	472	0	0	0	0
y tryp.	540	12	7.3	10	0
y trp/pep	521	16	12	0	0
mat buf	647	.4	3	3.7	0
mat tryp.	537	2	.1	0	0
mtrp/pep	601	10	4.6	6.9	0

TABLE 3 & 3.1. young buf = young corneal punches in buffer C; y tryp = young corneal punches in trypsin; y trp/pep = young corneal punches in trypsin for 24 hours followed by 3 peptidase solution for 72 hours; mat buf = mature corneal punches in buffer C; mat tryp. = mature corneal punches in trypsin; m trp/pep = mature corneal punches in trypsin for 24 hours followed by 3 peptidase solution for 72 hours; tot ug col = total ug of collagen present in corneal punch; ug @ 24, 48, 72, 96 hr = ug of collagen degraded by that time (TABLE 3) or at that time (TABLE 3.1); total % = percentage of total collagen degraded in 4 days.

TABLE 4: TOTAL DEGRADATION IN TRYP. & BUF. C AFTER BOILING

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr	total %
young buf	191	171	177.7	189.3	191	100
y tryp	207	194.6	200.6	204.6	207	100
mat buf	257	150	176	208	218	85
m tryp	273	247	264.5	270.8	273	100

TABLE 4.1: DAILY DEGRADATION IN TRYP. & BUF. AFTER BOILING

	tot ug col	ug @ 24hr	ug @ 48hr	ug @72hr	ug @ 96hr
young buf	191	171	6.7	11.6	1.3
y tryp	207	194.6	6	4.6	2.7
mat buf	257	150	26	32	9.8
m tryp	273	247	17.5	6.3	2

TABLE 4 & 4.1. young buf = boiled young corneal punches in buffer C; y tryp = boiled young corneal punches in trypsin; mat buf = boiled mature corneal punches in buffer C; m tryp = boiled mature corneal punches in trypsin; tot ug col = total ug of collagen present in corneal punch; ug @ 24, 48, 72, 96hr = ug of collagen degraded by that time (TABLE 4) or at that time (TABLE 4.1); total % = percentage of total collagen degraded in 4 days.

TABLE 5: TOTAL DEGRADATION IN ELASTASE & CATHEPSIN G

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr	total %
young buf	524	0	0	0	0	0
y elas	654	4	7.7	14	18.7	2.9
y cath	576	2	2.9	4.3	5.1	.88
mat buf	582	3.7	9.2	15	18.7	3.23
mat elas	421	0	1.5	1.9	3.8	.88
mat cath	718	9	19	27	36	5

TABLE 5.1: DAILY DEGRADATION IN ELASTASE & CATHEPSIN G

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr
young buf	524	0	0	0	0
y elas	654	4	3.7	6.3	4.7
y cath	576	2	.9	1.4	.8
mat buf	582	3.7	5.5	5.8	3.7
mat elas	421	0	1.5	.4	1.9
mat cath	718	9	10	7.8	8.8

TABLE 5 & 5.1. young buf = young corneal punches in buffer C; y elas = young corneal punches in elastase; y cath = young corneal punches in cathepsin G; mat buf = mature corneal punches in buffer C; mat elas = mature corneal punches in elastase; mat cath = mature corneal punches in cathepsin G; tot ug col = total ug of collagen present in corneal punch; ug @ 24, 48, 72, 96hr = ug of collagen

degraded by that time (TABLE 5) or at that time (TABLE 5.1); total % = percentage of total collagen degraded in 4 days.

TABLE 6: TOTAL DEGRADATION IN PLASMIN & CHYMOTRYPSIN

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr	total %
young buf	578	0	0	0	0	0
y plasmin	601	.6	4.6	9.6	20.6	3.4
y chymo	495	5.5	11.5	12.7	19	3.8
mat buf	663	1.9	6.9	13.5	24.5	3.7
mplasmin	545	0	0	0	0	0
m chymo	486	13.6	36.4	46.6	63	13

TABLE 6.1: DAILY DEGRADATION IN PLASMIN & CHYMOTRYPSIN

	tot ug col	ug @ 24hr	ug @ 48hr	ug @ 72hr	ug @ 96hr
young buf	578	0	0	0	0
y plasmin	601	.6	4	5	11
y chymo	495	5.5	6	1.2	6.4
mat buf	663	1.9	5	6.6	11
mplasmin	545	0	0	0	0
m chymo	486	13.6	22.8	10.2	16.5

TABLE 6 & 6.1. young buf = young corneal punches in buffer C; y plasmin = young corneal punches in plasmin; y chymo = young corneal punches in chymotrypsin; mat buf = mature corneal punches in buffer C; m plasmin = mature corneal punches in plasmin; m chymo = mature corneal punches in chymotrypsin; total ug col = total ug of collagen present in corneal punch; ug @ 24, 48, 72, 96hr = ug of collagen degraded by that time (TABLE 6) or at that time (TABLE 6.1); total % = percentage of total collagen degraded in 4 days.

TABLE 7: TOTAL DEGRADATION IN PROTEASES IN 18 HOURS

	tot ug col	ug @ 6hr	ug @ 12hr	ug @ 18hr	total %
y 3 pep	890	0	0	0	0
y elas	1194	0	3.2	3.2	.3
y cath	949	0	.9	.9	.1
y plas	956	2	6.7	6.7	.7
y tryp	945	1.2	11.6	11.6	1.2
y buf	1077	1.7	6.4	6.4	.6
m 3 pep	1180	0	0	0	0
m elas	1223	1.3	1.3	1.3	.1
m cath	1228	0	0	0	0
m plas	1230	2.9	2.9	2.9	.2
m tryp	1234	2	2	2	.17
m buf	1174	3.6	3.6	3.6	.3

TABLE 7.1: HOURLY DEGRADATION IN PROTEASES IN 18 HOURS

	tot ug col	ug @ 6hr	ug @ 12hr	ug @ 18hr
y 3 pep	890	0	0	0
y elas	1194	0	3.2	0
y cath	949	0	.9	0
y plas	956	2	4.7	0
y tryp	945	1.2	10.4	0
y buf	1077	1.7	4.7	0
m 3 pep	1180	0	0	0
m elas	1223	1.3	0	0
m cath	1228	0	0	0
m plas	1230	2.9	0	0
m tryp	1234	2	0	0
m buf	1174	3.6	0	0

TABLE 7 & 7.1. y 3 pep = young corneal punches in 3 peptidase solution; y elas = young corneal punches in elastase; y cath = young corneal punches in cathepsin G; y plas = young corneal punches in plasmin; y tryp = young corneal punches in trypsin; y buf = young corneal punches in buffer C solution; m 3 pep = mature corneal punches in 3 peptidase solution; m elas = mature corneal punches in elastase; m cath = mature corneal punches in cathepsin G; m plas = mature corneal punches in plasmin; m tryp = mature

corneal punches in trypsin; m buf = mature corneal punches in buffer C; tot ug col = total ug of collagen present in corneal punch; ug @ 6, 12, 18hr = ug of collagen degraded by that time (TABLE 7) or at that time (TABLE 7.1); total % = percentage of total collagen degraded in 18 hours.

TABLE 8: TOTAL SOL OF MAT PUNCHES IN BUF C AFTER BOIL

	0hr	6hr	12h	18h	24h	30h	36h	42h	48h	60h	66h	72h	t%
mcn	0	0	0	2.3	2.3	2.3	2.3	2.3	2.3	2.3	13	13	1
min	0	0	0	0	0	2.7	2.7	2.7	2.7	2.7	13	13	1
men	0	0	0	7.7	7.7	7.7	10	10	10	10	42	42	4
mcb	164	278	306	314	314	317	322	322	322	333	333	333	33
mib	120	279	297	297	297	297	297	297	297	297	297	297	32
meb	113	198	206	206	209	211	215	215	215	226	226	226	22

TABLE 8.1: DAILY SOL OF MAT PUNCHES IN BUF C AFTER BOIL

	0hr	6hr	12h	18h	24h	30h	36h	42h	48h	60h	66h	72h
mcn	0	0	0	2.3	0	0	0	0	0	0	10.7	0
min	0	0	0	0	0	2.7	0	0	0	0	10.1	0
men	0	0	0	7.7	0	0	2.4	0	0	0	32	0
mcb	164	114	28	7.8	0	3	5	0	0	11	0	0
mib	120	159	18	0	0	0	0	0	0	0	0	0
meb	113	85	8.3	0	3	2	4	0	0	11	0	0

TABLE 8 & 8.1. mcn = mature center corneal punches, non-boiled in buffer C; min = mature intermediate corneal punches, non-boiled in buffer C; men = mature edge corneal punches, non-boiled in buffer C; mcb = mature corneal punches, boiled in buffer C; mib = mature intermediate corneal punches, boiled in buffer C; meb = mature edge corneal punches, boiled in buffer C; 0, 6, 12, 18, 24, 30, 36, 42, 48, 60, 66, 72hr = ug of collagen solubilized by that time (TABLE 8) or at that time (TABLE 8.1); t % = percentage of total collagen solubilized in 3 days.

TABLE 9: TOTAL SOL OF YOUNG PUNCHES IN BUFFER C AFTER BOIL

	0hr	6hr	12h	18h	24h	30h	36h	42h	48h	60h	66h	72h	t%
ycn	0	0	0	0	0	0	0	0	0	0	0	0	0
yin	0	0	0	2	2	2	2	·13	13	13	14	14	1
yen	0	0	0	0	0	0	0	0	0	0	0	0	0
ycb	213	432	432	440	440	440	440	440	440	440	440	440	64
yib	275	665	701	711	711	713	715	715	715	718	718	718	63
yeb	184	463	490	490	490	490	490	490	490	490	490	490	73

TABLE 9.1: DAILY SOL OF YOUNG PUNCHES IN BUF C AFTER BOIL

	0hr	6hr	12h	18h	24h	30h	36h	42h	48h	60h	66h	72h
ycn	0	0	0	0	0	0	0	0	0	0	0	0
yin	0	0	0	2	0	0	0	11	0	0	14	0
yen	0	0	0	0	0	0	0	0	0	0	0	0
ycb	213	219	0	8	0	0	0	0	0	0	0	0
yib	275	390	36	10	0	1.5	2.4	0	0	32	0	0
yeb	184	279	27	0	0	0	0	0	0	0	0	0

TABLE 9 & 9.1. ycn = young center corneal punches, non-boiled in buffer C; yin = young intermediate corneal punches, non-boiled in buffer C; yen = young edge corneal punches, non-boiled in buffer C; ycb = young center corneal punches, boiled in buffer C; yib = young intermediate corneal punches, boiled in buffer C; yeb = young edge corneal punches, boiled in buffer C; 0, 6, 12, 18, 24, 30, 36, 42, 48, 60, 66, 72 hr = ug of collagen solubilized by that time (TABLE 9) or at that time (TABLE 9.1); t % = percentage of total collagen solubilized in 3 days.

TABLE 10: TOTAL SOLUBILIZATION OF COLLAGEN IN BUFFER C

	ug in 24hr	ug in 72hr	ug in 96hr	ug in 120	total ug
y center	0	0	0	0	1727
y inter	0	0	0	0	1553
y edge	0	0	0	0	1463
m center	0	0	0	0	2426
m inter	0	0	0	0	1835
m edge	0	0	0	0	1957

TABLE 10. y center = young center corneal punch in buffer C; y inter = young intermediate corneal punch in buffer C; y edge = young edge corneal punch in buffer C; m center = mature center corneal punch in buffer C; m inter = mature intermediate corneal punch in buffer C; m edge = mature edge corneal punch in buffer C; total ug = total ug of collagen present in corneal punch; ug @ 24, 72, 96, 120hr = total ug of collagen solubilized by that time.

TABLE 11: SOL. OF PERIPHERAL 2mm RING OF 6mm CENTRAL PUNCH

	tot ug collagen	ug col sol @4d	% col sol in 4d
young 1	1009	0	0
young 2	562	6	1.1
young 3	887	0	0
young 4	552	6.6	1.2
mature 1	513	0	0
mature 2	929	1.3	1.4
mature 3	919	0	0
mature 4	646	1	.15

TABLE 11. young 1 = superior section of a 2mm peripheral ring of a 6mm central punch of young corneas; young 2 = medial section; young 3 = inferior section; young 4 = lateral section; mature 1 = superior section of a 2mm peripheral ring of a 6mm central punch of mature corneas; mature 2 = medial section; mature 3 = inferior section; mature 4 = lateral section; total ug col = total ug of collagen present in section of ring; ug col @ 4 d = ug of collagen solubilized at that time; % col sol in 4 d = percentage of total collagen solubilized in 4 days.

TABLE 12: TOTAL SOL AFTER BOILING W/ INHIBITOR VS. W/O

normal	ug in 24hr	tot ug col	inhibitor	ug in 24hr	tot ug col
young	343 (47%)	731	young	305 (39%)	787
mature	306 (31%)	994	mature	250 (27%)	924

TABLE 12. normal = those corneal punches boiled without inhibitor cocktail; young = young corneal punches; mature = mature corneal punches; inhibitor = those corneal punches boiled with inhibitor cocktail; ug in 24hr = ug of collagen solubilized by that time; tot ug col = total ug of collagen present in corneal punch.

TABLE 13: TOTAL COLLAGEN IN YOUNG CORNEAL PUNCHES

	total ug collagen
young #1 edge	1555 +/- 42
young #1 intermediate	1395 +/- 151
young #1 center	1575 +/- 23
young #2 edge	1884 +/- 104
young #2 intermediate	1862 +- 74
young #2 center	2022 +/- 21
young #3 edge	1539 +/- 76
young #3 intermediate	1699 +/- 105
young #3 center	1718 +/- 128

TABLE 13. young #1 edge = edge corneal punches from young animal eye 1; young #1 intermediate = intermediate corneal punches from young animal eye 1; young #1 center = center corneal punches from young animal eye 1; young #2 edge = edge corneal punches from young animal eye 2; young #2 intermediate = intermediate corneal punches from young animal eye 2; young #2 center = center corneal punches from young animal eye 2; young #3 edge = edge corneal punches from young animal eye 3; young #3 intermediate = intermediate corneal punches from young animal eye 3; young #3 center = center corneal punches from young animal eye 3; total ug collagen = average total ug of collagen present in corneal punches.

TABLE 14: TOTAL COLLAGEN IN MATURE CORNEAL PUNCHES

	total ug collagen
mature #1 edge	2104 +/- 33
mature #1 intermediate	1993 +/- 133
mature #1 center	2168 +/- 73
mature #2 edge	2010 +/- 102
mature #2 intermediate	1966 +/- 160
mature #2 center	1812 +/- 20
mature #3 edge	1722 +/- 123
mature #3 intermediate	2216 +/- 54
mature #3 center	1977 +/- 46

TABLE 14. mature #1 edge = edge corneal punches from mature animal eye 1; mature #1 intermediate = intermediate corneal punches from mature animal eye 1; mature #1 center = center corneal punches from mature animal eye 1; mature #2 edge = edge corneal punches from mature animal eye 2; mature #2 intermediate = intermediate corneal punches from mature animal eye 2; mature #2 center = center corneal punches from mature animal eye 2; mature #3 edge = edge corneal punches from mature animal eye 3; mature #3 intermediate = intermediate corneal punches from mature animal eye 3; mature #3 center = center corneal punches from mature animal eye 3; total ug collagen = average total ug of collagen present in corneal punches.

TABLE 15: TOTAL COLLAGEN IN CORNEAL PUNCHES

	total ug collagen
young edge	1659 +/- 113
young intermediate	1652 +/- 137
young center	1771 +/- 132
mature edge	1947 +/- 115
mature intermediate	2058 +/- 79
mature center	1986 +/- 103

TABLE 15. young edge = edge corneal punches from young animal eyes 1, 2, and 3; young intermediate = intermediate corneal punches from young animal eyes 1, 2, and 3; young center = center corneal punches from young animal eyes 1, 2, and 3; mature edge = edge corneal punches from mature animal eyes 1, 2, and 3; mature intermediate = intermediate corneal punches from mature animal eyes 1, 2, and 3; mature center = center corneal punches from mature animal eyes 1, 2, and 3; total ug collagen = average total ug of collagen present in corneal punches.

TABLE 16: DETER. OF FRAGMENTATION OF COL. AFT. DENATURATION

	ug col aft boil	ug col aft dial.	total ug col.
young	540	535	1311
mature	580	445	1634

TABLE 16. young = young corneal punches; mature = mature corneal punches; ug col aft boil = ug of collagen solubilized after denaturation by boiling; ug col aft dial. = ug of solubilized collagen remaining in sample after dialysis; total ug col. = total ug of collagen present in corneal punches.