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Collagenase is produced by both the normal and inflamed conjunctiva. The cell of origin is probably the epithelium. Inhibition could be carried out by the usual inhibitors. Partial purification of the enzyme from the inflamed tissue showed a discrete peak of activity at a molecular weight of 65,000. It is suggested that an abnormal production of collagenase by the conjunctiva may play a role in the formation of peripheral corneal ulcers and scleromalacia.

During the past five years, studies of corneal collagenolytic enzymes indicated that they cause the ulcers of the alkali-burned cornea and are probably partially involved in the pathogenesis of many other corneal ulcers.

Recently, a study on the pathogenesis of Mooren's ulcer identified a proteoglycanolytic enzyme and a collagenase produced by the conjunctiva adjacent to the ulcers. Because of the limited amount of tissue available in this study these enzymes were not characterized.

The present report characterizes a partially purified collagenase from rabbit conjunctiva.

Materials and methods. Albino rabbits weighing two to three kilograms were divided into four groups. The first group was anesthetized with intravenous pentobarbital and topical tetracaine hydrochloride. Complete Freund's adjuvant in 0.9 per cent NaCl (0.05 ml.) was injected through a 25-gauge needle into the subconjunctival space adjacent to the corneal limbus at the 12, 3, 6, and 9 o'clock positions. The animals were killed after 2, 4, 7, 10, 14, and 21 days and the inflamed conjunctiva was excised and tested for collagenolytic activity via tissue-culture assay.

The second group of rabbits was treated similarly as the first group except that all animals in this group were killed at seven days and their limbic conjunctiva was removed. Half of the tissues were tested for collagenolytic activity and the other half was used to harvest a collagenase.

The third group was treated identical to the second group except that the conjunctival epithelium was scraped off with the edge of a scalpel before the tissue was excised. Histologic sections of these tissues proved the epithelium was completely removed after scraping. The inflamed conjunctival stroma was then tested for collagenolytic activity.

The fourth group of rabbits was killed with intravenous pentobarbital and a ring of normal untouched limbic conjunctiva 4 mm. wide was excised. Part of the tissue was tested for collagenolytic activity and the remaining tissue was used to harvest a collagenase.

Harvest of collagenase. The excised conjunctiva from the inflamed and normal conjunctiva was cut into approximately 2 by 2 mm. pieces, washed with a mammalian Tyrode's streptomycin solution, and placed into sterile organ culture dishes. Care was taken to be certain that there were an equal number of normal versus inflamed pieces of conjunctiva and that they were incubated in separate dishes. The culture media were prepared from equal amounts of Tyrode's solution and Dulbecco's modified Eagle's Medium with 100 mg. per liter of streptomycin sulfate, and was equilibrated with 95 per cent oxygen and 5 per cent carbon dioxide prior to use. The tissues were incubated at 37°

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Fractions to be assayed for collagenase activity distilled water was added and the tubes were incubated for 24 to 48 hours in a moist atmosphere at 37° C. Following incubation, 0.1 ml. of distilled water was added and the tubes were centrifuged at 44,000 x g for 10 minutes. Aliquots of the supernatant (0.25 ml.) were pipetted into vials containing glass filter papers and dried overnight. The radioactivity was counted in a liquid scintillation counter in 1.0 ml. of POPP-1, 4-bismethyl-5-phenylloxazolyl-benzene, PPO-2, 5-diphenyloxazole, and toluene mixture.

**Purification of collagenase.** Collagenase activity was monitored in each step of purification on 0.2 per cent glycine-C14 collagen gels.

A saturated ammonium sulfate solution, pH 7.0, was added to the pooled culture media from the inflamed and normal conjunctiva so that the final saturation was 50 per cent. The precipitate containing the collagenase was dissolved in 50 mM Tris HCl buffer, pH 7.4, containing 5 x 10^-2 M CaCl2, and dialyzed against this buffer for six hours at 4° C. The enzyme solution (1.0 to 2.0 ml.) containing 6 to 8 mg. of protein was then applied to the columns.

Sephadex G-200 columns (1.6 by 82.0 cm.) were equilibrated with 50 mM Tris HCl, pH 7.4, containing 4 mM CaCl2. The flow-rate was 16 ml. per hour. The lyophilized harvest media (10 mg.) were dissolved in 1.5 ml. of 50 mM Tris HCl, pH 7.4, containing 4 mM CaCl2 and applied to the columns, and 3.4 ml. fractions were collected. The temperature was maintained at 4° C. The protein content was approximated at 280 nm. on a Beckman DB-G spectrophotometer. Every five fractions were concentrated under vacuum or were lyophilized, and were assayed for collagenase activity. The concentrated fractions contained 30 to 40 µg per 10 µl of solution.

**Viscometry.** Viscometry measurements were made in Ostwald viscometers with water flow times of 49 and 53 seconds at 27° C. The collagen solution (0.1 per cent) was prepared for viscometry by adding 0.05 M Tris HCl buffer, pH 7.4, containing 0.14 M NaCl. Measurements of collagen breakdown with the harvest media from normal and inflamed conjunctiva (200 µg of protein) were taken over a five-hour period. The initial viscosity (nsp.) was 7.0. Control viscosity measurements of viscosity without harvest media were run simultaneously.

**Optical rotation.** The optical rotation of solutions of reconstituted collagen, with and without the conjunctival harvest media, was determined using the MSP-3 photoelectric spectrophotometer at 25° C. at 589 mp.
Fig. 2. Estimation of the molecular weight of the conjunctival collagenase by gel filtration on Sephadex G-200.

Estimation of molecular weight of collagenase. The molecular weight of the partially purified inflamed conjunctival collagenase was calculated using the method of Andrews. Aldolase, chymotrypsinogen A, bovine serum albumin, ribonuclease A, and blue dextran 2,000 (Pharmacia, Piscataway, N. J.) were used as standards. The protein content of the eluted standards was approximated at 280 nm.

Inhibition of conjunctival collagenase. Cysteine, 10⁻³ M; NaEDTA, 10⁻³ M; penicillamine, 10⁻³ M and 10⁻⁴ M; and serum 1:100 were separately added to the partially purified collagenases from the inflamed conjunctiva and assayed for collagenase activities at pH 7.4.

Results. Within 24 hours after injection of Freund's adjuvant the conjunctiva became markedly edematous with dilated blood vessels. Tissue culture assay of the excised inflamed conjunctiva showed significant collagen gel lysis (5 to 10 mm in diameter) by 17 of the 20 specimens from four to 14 days after injection.

Tissue culture assay of the normal conjunctiva showed significant collagen gel lysis in 11 of the 20 specimens.

Collagenase activity appeared only in the precipitate of the 50 per cent saturated solution of the harvest media from both the inflamed and the normal conjunctiva (Table I). Gel infiltration of the inflamed conjunctival harvest media demonstrated one discrete reproducible peak of collagenolytic activity (Fig. 1). This partially purified collagenase had a molecular weight of 65,000 (Fig. 2).

Gel infiltration of the harvest media from the normal conjunctiva yielded relatively low net collagenolytic activity. Consequently, the peak of activity, though similar to the inflamed tissue, was not considered reliable.

The partially purified enzyme from both normal and inflamed conjunctiva caused a reduction in the viscosity of a collagen solution of approximately 40 per cent. Simultaneous controls showed a decrease in viscosity of less than 5 per cent. The optical rotation of the collagen solution did not change significantly before or after the viscosity experiments. When the viscosity reaction mixture was tested on polyacrylamide gel electrophoresis the resultant disc electrophoresis patterns were typical of mammalian collagenases (Fig. 3).

There was no significant change in viscosity of a solution of corneal proteoglycan after mixing with (NH₄)₂SO₄ precipitated harvest media.

The inhibitors of conjunctival collagenase are listed in Table I.
Discussion. The results of these studies show that a collagenase is produced by both the normal and the injured rabbit conjunctiva. The inflamed tissue seemed to produce relatively greater quantities of the enzyme. This was indicated by the tissue culture assays and by the greater amounts of radioactivity released when the two harvest and purified media were assayed for collagenolytic activity (Table 1).

Partial purification of the enzyme produced by the inflamed tissue showed a discrete peak of activity which had a molecular weight of 65,000. Attempts at purifying the normal conjunctival enzyme indicated a similar peak of activity but there was too little enzymatic activity to be certain.

The collagenase from the inflamed conjunctiva was inhibited by penicillamine in addition to the usual inhibitors.

The cell origin of the enzyme was probably the epithelium since its removal by scraping prevented collagen gel lysis in the tissue-culture assay.

It may be speculated from the present and the Moorén's ulcer studies that conjunctival collagenases are involved in the formation of peripheral corneal ulcers and perhaps scleromalacia.

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Tear lactoferrin: a bacteriostatic and complexing protein. R. M. BROEKHUYSE.

The presence of lactoferrin in human tears is established by immunoochemical techniques. Together with tear pre-albumin, IgA, and lysozyme they constitute the major tear proteins. Lactoferrin added to tears binds to IgA, IgG, and serum albumin and alters their electrophoretic mobility. On agarose and agar gels it has a different electrophoretic behavior, which together with its complexing properties offers several possibilities for misinterpretation of immunoochemical results. Lactoferrin has an apparent molecular weight of approximately 82,000 as established by dodecyl sulfate-polyacrylamide gel electrophoresis. It has bacteriostatic properties, presumably due to its ability to make certain metals unavailable for microorganisms.

Protein composition of tears has been extensively investigated during the past ten years. Sapse and co-workers identified serum albumin, ceruloplasmin, transferrin, IgA, IgG, lysozyme, and tear-prealbumin in human tears. They paid special attention to the function and quantitation of lysozyme and the immunoglobulins in human as well as in rabbit tears.4 The clinical significance of tear protein analyses was further demonstrated by van Bysterveld and Liotet and Rouchy.6 Most studies have been concerned with the protecting function of tears or with the changing protein composition in pathologic conditions.

Recent studies increasingly reveal the complex nature of the tear film in which spreading factors,6 cholesterol esters, mixed waxes,6 proteinase inhibitor,7 and an oily layer on the surface10 have been demonstrated. The protecting function seems to be predominantly twofold. Immunoglobulins and lysozyme eliminate bacteria and penetrating impurities, while other components assure its physical integrity and create, therefore, the optimal environment for the underlying tissues.

Recently, two other factors were found: a nonlysozyme antibacterial factor was demonstrated in human tears,11 and during immuno-electrophoretic analyses of tears we detected the presence of lactoferrin, a transferrin-like protein, previously found in various physiologic fluids and in granulocytes.12 This protein displays such remarkable protein-binding properties13 and bacteriostatic activity,12 that we undertook a study in order to confirm its presence in tears and its influence on the immunoelectrophoretic pattern of human tear proteins.