

Chelation in experimental *Pseudomonas* keratitis

LOUIS A. WILSON

Division of Ophthalmology, Medical College of Georgia

In contrast to its occasional occurrence during the first half of the 20th century (Joy, 1942), *Pseudomonas aeruginosa* to-day ranks as a common, if not the most common, cause of bacterial corneal ulcer in the United States of America. Even with prompt laboratory diagnosis and use of adequate and specific antimicrobial therapy, the rapidly destructive lesion caused by this bacterium frequently results in severe visual impairment and often in corneal perforation with its attending complications. Such perforation is often predictable, in the experience of the author, when a spreading colliquative necrosis of the cornea continues to occur following conversion of daily cultures from positive to negative.

It has been suggested that this rapid corneal destruction may be the result of an extremely potent bacterial proteolytic enzyme (Fisher and Allen, 1958a, b; Fisher, 1958). This has been described as being intracellularly synthesized by *Pseudomonas aeruginosa*, released and extracellularly activated by available calcium cations (Morihara, 1963). The ability to split certain synthetic hexapeptides in a highly specific manner, plus its apparent action on the corneal stroma which is largely collagen, suggests further that this metallo-enzyme is in fact a collagenase (Schoellmann and Fisher, 1966).

Apart from *Pseudomonas aeruginosa*, only three groups of microbial organisms have been shown to produce such an enzyme (MacLennan, Mandl, and Howes, 1953; Rippon, 1968a, 1968b) (Table I).

Table I *Microbial organisms producing collagenase*

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- (1) *Clostridium histolyticum*
 - (2) *Pseudomonas aeruginosa*
 - (3) *Streptomyces madurae* (Nocardia)
 - (4) *Trichophyton schoenleinii*
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All such collagenases demonstrate certain common features (Table II, overleaf). Of these properties, the ability of low concentrations of the disodium salt of ethylenediamine tetra-acetic acid (Na_2EDTA) to inhibit enzymatic hydrolysis of the substrate collagen appeared to have some therapeutic possibilities in the management of *Pseudomonas* keratitis. It was the purpose of this investigation to determine experimentally whether this might be so.

Table II Characteristics of microbial collagenase

Organism	Ca ⁺² Metallo-enzyme	Inhibition Na ₂ EDTA	Inhibition cysteine	Enzyme disrupters
<i>Clostridium histolyticum</i>	+	+ .. R	+ .. NR	Urea Heavy metals
<i>Pseudomonas aeruginosa</i>	+	+ .. R	+ .. NR	Urea Heavy metals KMnO ₄
<i>Streptomyces madurae</i>	+	+ .. R	+ .. NR	Urea Heavy metals
<i>Trichophyton schoenleinii</i>	+	+ .. NR	+ .. NR	Urea Heavy metals

R = Reversible inhibition
NR = Nonreversible inhibition

Material and methods

Four strains of *Pseudomonas aeruginosa* isolated from clinical cases of keratitis were evaluated for their ability to produce proteolysis by inoculation onto a special protein* and gelatin medium. The same isolates were also inoculated onto this medium containing Na₂EDTA in concentrations of 2.5×10^{-3} M, 5×10^{-3} M, and 10^{-2} M respectively. Incubation was at 27°C. and the cultures were observed for evidence of proteolysis at 24, 36, and 48 hrs. This was determined in gelatin by liquefaction while on the opaque protein media by a clearing to near transparency extending outwards from the inoculation site.

Cell-free filtrates of the four strains grown for 36 hours in 5 per cent. peptone broth, both with and without 10^{-2} M, Na₂EDTA, were obtained using a Millipore (0.22u) filtration system. Collagen strips† were then incubated in these filtrates at 37°C. and observed at 24, 36, and 48 hrs for gross evidence of hydrolysis. No attempt was made to determine the amino acid content of the proteolytic end products.

Since, during the *in vitro* phase, all of the *Pseudomonas* strains behaved in an identical fashion, one was chosen at random to continue the study.

Using techniques previously described by earlier investigators (Moriyama, 1963), a crude enzyme extract was obtained from cell-free filtrates of this strain grown for 36 hrs in a 5 per cent. peptone broth. The enzyme was reconstituted with phosphate buffer (pH 7.2) and 1/10 of a millilitre injected intrastromally in the right cornea of six adult albino rabbits. The enzyme extract was also reconstituted in 10^{-2} M Na₂EDTA buffered to pH 7.0, and an identical amount was injected similarly into the left cornea of the same animals. They were then observed at 12 and 24 hrs for evidence of liquefactive corneal necrosis.

To ascertain the effect of Na₂EDTA in the face of actual infection, both corneae of 24 adult albino rabbits were inoculated with overnight cultures of *Pseudomonas aeruginosa* grown in tryptic soy broth, using the Hessburg technique of running a contaminated 6-0 black silk suture through the superficial stroma (Hessburg, Truant, and Penn, 1963). Between 18 and 20 hrs after inoculation, the animals were divided into two groups of twelve each for treatment. In all of the 24 animals, both eyes were treated with a gentamicin-carbanicillin mixture (8 mg. gentamicin-4 mg. carbanicillin per ml.) in 1 per cent. methylcellulose, administered hourly from 8 a.m. to 8 p.m. for 7 days, then four times daily for another week. In one group of twelve animals, the left eye was additionally treated with a 10^{-2} M solution of Na₂EDTA in 1 per cent. methylcellulose (pH 7.0) four times daily for 2 weeks; in the other group of twelve a 0.5 per cent. Na₂EDTA ointment was applied to the left cornea four

*Protein medium 0.2 per cent. casein, 0.3 per cent. beef extract, 0.5 per cent. tryptone, 0.1 per cent. glucose, and 1.5 per cent. agar (pH 6.7).

†Collagen strips 3 × 2 mm. strips of rabbit achilles tendon aseptically obtained and bacteriologically sterile.

times daily for the same period of time. In all animals both corneae were cultured for *Pseudomonas* before starting treatment and in the morning daily thereafter for 14 days. All corneae were observed daily for progress of the infection as manifested by corneal infiltration, extent of liquefactive necrosis (softening and/or loss of stroma), descemetocoele, or perforation (Tables III and IV for grading).

Table III Evaluation Day 7 Na_2EDTA 10^{-2}M in 1 per cent. methylcellulose

Rabbit no.	Antibiotics only Right eye	Antibiotic + Na_2EDTA 10^{-2}M Left eye
1	4	2
2	4	2
3	3	1
4	4	3
5	3	2
6	3	2
7	4	2
8	3	1
9	3	3
10	4	2
11	4	2
12	4	2
Average	3.58	2.00

Table IV Evaluation Day 7 Na_2EDTA 0.5 per cent. Ointment

Rabbit no.	Antibiotics only (Right eye)	Antibiotic + 0.5 per cent. EDTA ointment (Left eye)
1	4	3
2	3	2
3	3	2
4	4	2
5	4	2
6	4	1
7	3	2
8	4	2
9	4	1
10	3	3
11	4	2
12	4	2
Average	3.66	2.00

Grade 0 No ulceration, 0 mm.

Grade 1 Central infiltration, superficial necrosis 1-3 mm.

Grade 2 Central infiltration, superficial necrosis 3-5 mm.

Grade 3 More than one-half cornea infiltrated, extensive necrosis, loss of stroma, descemetocoele

Grade 4 Entire cornea infiltrated, extensive necrosis, loss of stroma, perforated

Results

During the *in vitro* phase all strains behaved in an identical fashion, causing a rapid enzymatic clearing of the opaque protein media and liquefaction of the gelatin within 24 hrs. At a concentration of $2.5 \times 10^{-3}\text{M}$, Na_2EDTA had no sustained inhibitory effect on proteolysis, either on the protein media or on gelatin, and at $5 \times 10^{-3}\text{M}$ Na_2EDTA , there was inhibition of gelatin liquefaction, but proteolysis was noted at 48 hrs on the opaque protein media. The media incorporating 10^{-2}M Na_2EDTA exhibited no evidence of proteolysis even at the end of a week. The addition of sterile 10^{-3}M calcium chloride at this time resulted in the appearance of proteolytic activity within 24 hrs.

In the cell-free filtrates with no Na_2EDTA , hydrolysis of the collagen strips was partial at 24 hrs and complete in from 36 to 48 hrs. However, on incubation with the filtrates containing 10^{-2}M Na_2EDTA , there was no apparent enzymatic digestion of the strips at the end of 48 hrs. As with the solid media, proteolytic activity was noted within 24 hrs after the addition of calcium chloride.

On intrastromal injection of 1/10 ml. of enzyme extract into the right cornea of six rabbits, there occurred a marked colliquative necrosis within 12 hrs in each animal. Apart from the absence of a mucopurulent conjunctival discharge, this looked quite like a *Pseudomonas* infection, although cultures were negative (Fig. 1A). The addition of 10^{-2}M Na_2EDTA to the enzyme preparation before intrastromal injection into the left cornea of the same six animals did not prevent a stromal reaction as manifested by central

infiltration and superficial ulceration, but there was evident inhibition of the extensive liquefying necrosis noted in the right cornea (Fig. 1B).

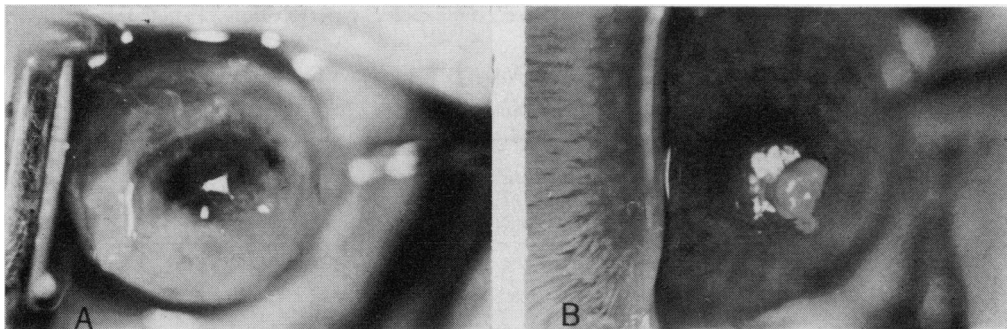


FIG. 1 (A) Right cornea 12 hrs after intrastromal injection of 0.1 ml. enzyme extract.

(B) Left cornea of same animal 12 hrs after intrastromal injection of enzyme in $10^{-2}M$ Na_2EDTA

All of the 24 animals inoculated exhibited evidence of bilateral corneal infection between the 18th and 20th hour after inoculation (Fig. 2A and B) and at this time, the particular form of therapy was initiated. In the right cornea of both groups treated with antibiotics alone, a liquefactive necrosis was noted to begin between the 22nd and 24th hour which progressed concentrically and in depth from the point of inoculation to involve most or all

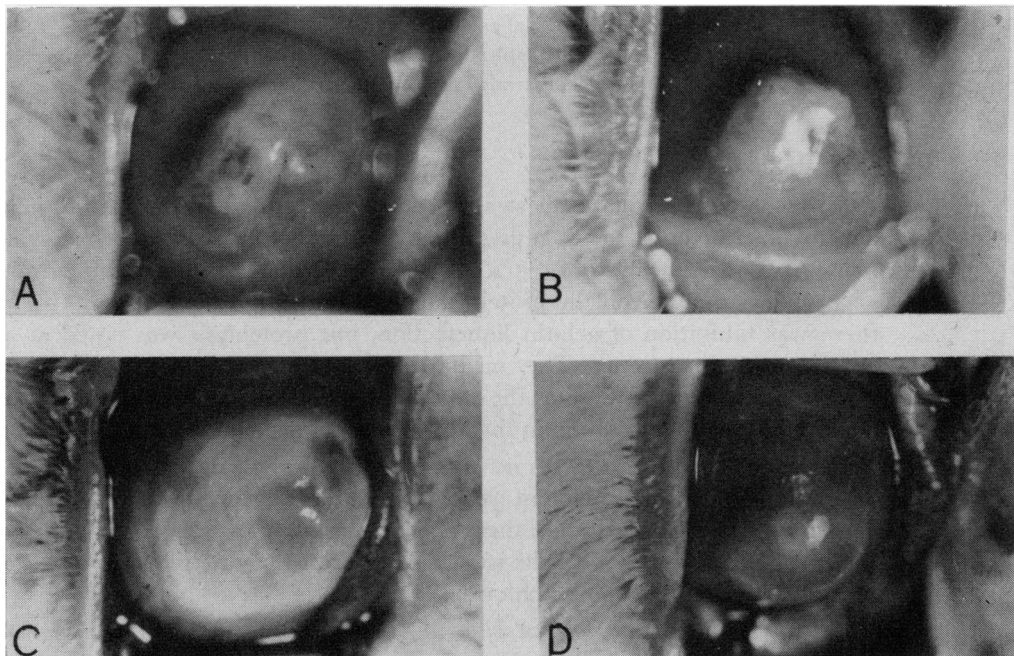


FIG. 2 *Representative animal.*

(A) Right eye 20 hrs after inoculation

(B) Left eye 20 hrs after inoculation

(C) Right eye on Day 7 treated with antibiotics only

(D) Left eye on Day 7 treated with antibiotics plus $10^{-2}M$ Na_2EDTA in 1 per cent. methylcellulose

of the cornea, reducing its consistency from firm to gel-like. By the seventh day, fifteen right corneae from the total of 24 had perforated (Tables III and IV, Fig. 2C). This occurred in the face of specific antibiotic therapy and in spite of the fact that by the third day of treatment, all cultures from these eyes were negative for *Pseudomonas*.

In the left cornea of the twelve rabbits in group one treated additionally with 10^{-2} M Na_2EDTA drops four times daily, the results were somewhat different. By the fourth day there was ulceration at the site of inoculation and a spreading concentric infiltration of 5 mm. or more in most of the animals. However, in only two did a gel-like consistency develop and this was limited in comparison to the extensive destruction noted in the fellow right eye. The appearance of a descemetocoele was not noted in any of these left corneae by Day 7. As with the right eye, negative cultures were obtained by the third day of treatment (Table III, Fig. 2D).

In the second group of twelve rabbits, the left cornea was treated with 0.5 per cent. Na_2EDTA ointment four times daily in addition to the antibiotic drops. The results in these animals were much the same as in those eyes treated with Na_2EDTA drops. In no instance was perforation noted and ten of the twelve corneae maintained a firm stromal consistency (Table IV).

In all animals, the second 7 days of therapy on both sides appeared to be periods of resolution from the initial insult. In the 24 right corneae there was dense scarring, extensive neovascularization, and in those which perforated adherent leucoma formation. On the left side neovascularization, although present, was less; and while there was central scarring, the peripheral cornea seemed to clear somewhat. At the end of one month, the difference between the two sides was quite apparent (Fig. 3).

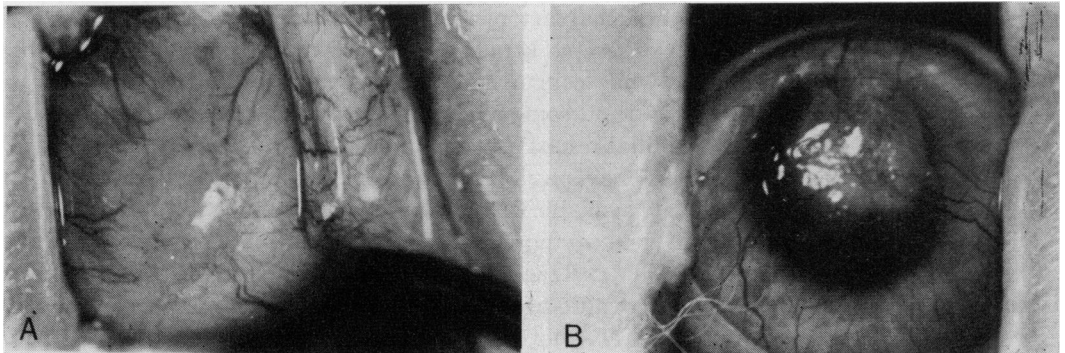


FIG. 3 *Representative animal 1 month after inoculation*

- (A) Right eye treated with antibiotics only
- (B) Left eye treated with antibiotics and 10^{-2} M Na_2EDTA

Discussion

From the results of this study plus his clinical experience, the author concurs with the observation of Hessburg and others (1963) that both in experimental and in most clinical instances of *Pseudomonas aeruginosa* keratitis, the majority of evident damage is accomplished by the fourth day of active disease. The continuation of this damage in the face of negative cultures would suggest that it is caused by more than the simple presence of replicating bacteria.

Although in this series of experimental infections only one strain of *Pseudomonas aeruginosa* was used, studies in this laboratory have since shown that, when any of our clinical cornea isolates are inoculated into the rabbit cornea and treated with antibiotics alone, the results are almost identical.

Several years ago Fisher (1958) and Fisher and Allen (1958a, b) suggested that a *Pseudomonas*-elaborated proteolytic enzyme may be the cause of major damage in such corneal infections. Since then, Morihara and Tsuzuki (1964) have shown that *Pseudomonas aeruginosa* does indeed produce such an enzyme, a peptidohydrolase. They have also demonstrated that it is a calcium activated metallo-enzyme. More recent studies have indicated that this enzyme may be a collagenase akin to that produced by *Clostridium histolyticum* (Schoellmann and Fisher, 1966).

This investigation accepts the results of these earlier studies, and no effort was made to duplicate them or prove that this enzyme is specifically a collagenase. However, its results confirm the proteolytic effect of *Pseudomonas aeruginosa* on various media and collagen strips. That Na_2EDTA can block such action without interfering with bacterial growth and that such inhibition is reversible on the addition of dilute calcium chloride solution lends support to the concept that this enzyme is calcium dependent. The ability of a crude extract of this enzyme when injected intrastromally to mimic an infection in the absence of bacteria suggests further that it is this bacterial product which is primarily responsible for the extensive corneal destruction seen in *Pseudomonas aeruginosa* keratitis.

The concept of employing a chelating agent *in vivo* to block the destructive effect of bacterial enzyme systems is not a new one. Lynch and Moskowitz (1968) have reported on the ability of various chelators administered parenterally to protect animals against otherwise lethal doses of *Clostridium perfringens* alpha toxin, a lecithinase. This enzyme, considered to be the lethal factor in gas gangrene, is apparently a zinc metallo-enzyme.

In instances of *Pseudomonas* keratitis, it seemed that, if one could inhibit the action of the bacterial protease while concurrently administering antibiotics to eliminate the enzyme source, the therapeutic outcome might be somewhat better than is currently the case. On the basis of these initial results it would appear that this may be possible. The protection afforded by EDTA may possibly be two-fold. Similar to its effect in various protein media, it can chelate available free calcium cations (as noted in Fig. 4, Reaction 1), making them unavailable for enzyme activation. The unactivated enzyme may then bind to stromal lamellae (Fig. 4, Reaction 2) but, unactivated by calcium, no proteolysis occurs. It may also undergo autodigestion. As an additional protective measure it is quite probable that Na_2EDTA forms a calcium chelate-enzyme complex with the already activated enzyme (Fig. 4, Reaction 4) and thus makes it unavailable for proteolysis (Morihara and Tsuzuki, 1964).

The kinetics of enzyme-substrate interaction and constant replenishment of calcium from tears, cornea, and aqueous, would dictate that, in the living cornea, complete inhibition of stromal digestion in the presence of infection and enzyme production is not possible. This may explain the modified necrosis noted in the left Na_2EDTA -treated corneae of both groups of animals.

There are other methods of inhibiting this proteolytic activity. However, our experience with enzyme disruptors such as 6M urea and heavy metals have not met with much success, their application to the cornea frequently being as destructive as the infection itself, or more so. Cysteine would appear to possess some therapeutic potential, but so far our results *in vivo* have not been as productive as with Na_2EDTA . The N-acetyl derivative of cysteine, available commercially as a mucolytic agent, had no effect at all. We are now

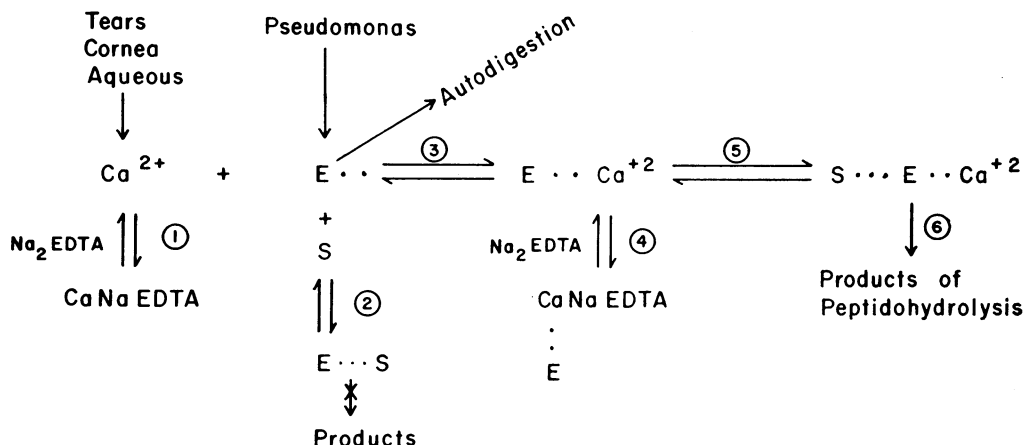


FIG. 4 Schematic representation of possible action of Na_2EDTA in inhibiting enzymatic hydrolysis of stromal collagen

investigating the use of more effective polycarboxylic amino acids as calcium chelators in an effort to inhibit this colliquative stromal necrosis in *Pseudomonas keratitis*, and experimentally at least several appear quite promising.

Summary

Clinical isolates from pseudomonal infections of the human cornea were evaluated for their ability to produce proteolysis, both *in vitro* and *in vivo*. A crude enzyme preparation was obtained from cell-free filtrates of *Pseudomonas aeruginosa* grown for 36 hrs in a 5 per cent. peptone broth. When a minute amount of this enzyme was injected into the corneal stroma of the experimental animal, a necrosis mimicking a clinical infection was noted to occur within 12 hrs. This necrosis was inhibited to a considerable degree by the addition of 10^{-2}M disodium EDTA. Similarly experimental infections in the rabbit cornea, when treated with appropriate antibiotics and a topical application of disodium EDTA, were noted to resist the extensive colliquative necrosis seen in similar corneae treated with antibiotics only.

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