

## HYALURONIDASE AS A SPREADING FACTOR IN THE CORNEA\*

BY

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THE permeability of the connective tissue throughout the body is controlled by the state of the interfibrillar ground substance. This ground substance has several components in nature, some protein, some mucopolysaccharide; the latter are the important factors in controlling the permeability of the tissue since, being highly polymerized, they form a viscous barrier to the diffusion of particles of colloidal dimensions. Skin contains two mucopolysaccharides, hyaluronic acid and chondroitin sulphate, in the ground substance of the dermis, while the cornea is stated to contain hyaluronosulphate. Hyaluronic acid and hyaluronosulphate can be broken down (depolymerized) by an enzyme hyaluronidase found in the filtrates of certain bacteria and in extracts of testis. The enzyme preparations of testis have also the power of depolymerizing chondroitin sulphate. Intradermal injections of hyaluronidase facilitate the diffusion of dyes, viruses, or toxins, by breaking down the viscous barrier offered by hyaluronic acid and this effect is the basis of the "spreading reaction".

The mucopolysaccharide of ox cornea was characterized by Meyer and Chaffee (1940) as hyaluronosulphate (a sulphate ester of hyaluronic acid) on the basis of its composition and because it was hydrolysed by hyaluronidase preparations from filtrates of the pneumococcus and testis. They suggested that the normal transparency of the cornea and its lack of blood vessels depended on the presence of hyaluronosulphate and showed that these characteristics were abolished by injections of hyaluronidase.

Wislocki, Bunting, and Dempsey (1947) found that the metachromatic staining reaction of cornea with toluidene blue was not abolished by treatment with testicular hyaluronidase, but drew no conclusions from this observation. It is possible that a small decrease in the intensity of staining would pass unnoticed under the conditions used by these authors. A further investigation of the nature of the mucopolysaccharides of the cornea was clearly required, and it was decided as a first step to assess the activity of hyaluronidase as a spreading factor in this tissue. Meyer had obtained a positive reaction using pneumococcal and testis enzymes.

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It may be stated that a positive spreading reaction in a tissue using hyaluronidase provides strong evidence for the presence of the substrate (hyaluronic acid or hyaluronosulphate) in that tissue, but this is not conclusive since no hyaluronidase preparations have been completely purified. A negative spreading reaction with a hyaluronidase preparation provides evidence for the absence of the substrate in that tissue provided that the conditions for the spreading reaction hold—that the mucopolysaccharide alone acts as a barrier to diffusion.

The subject is also of importance since clinical uses of the spreading reaction have recently been suggested (Gaisford and Evans, 1949; Atkinson, 1949), and hence a knowledge of the activity of hyaluronidase as a spreading factor in the cornea is of value apart from the evidence it provides of the nature of the mucopolysaccharides.

## Methods

*Testicular Hyaluronidase* was prepared from bovine testis according to the methods of Madinaveitia (1941). In one case an acetone powder was prepared from the aqueous extract of testis, in the other the aqueous extract was treated directly. The solution resulting from fractionation was dialysed against 0.9 per cent. aqueous NaCl.

*Cl. Welchii Hyaluronidase* was prepared according to the directions given for "crude hyaluronidase" by Rogers (1946). Hyaluronic acid from ox vitreous body was added to the growth medium.

*Streptococcal Hyaluronidase* was prepared according to the method of Rogers (1946) for "crude hyaluronidase". The filtrate was from a culture of a strain of streptococcus haemolyticus group A.

*Pneumococcal Hyaluronidase*. A type III pneumococcus was grown in a serum broth containing hyaluronic acid. The filtrate was not fractionated.

*Haemoglobin Solution*. Rabbit blood corpuscles were washed six times with 1 per cent. NaCl, laked with distilled water, and centrifuged, and the supernatant was dialysed against 0.9 per cent. NaCl.

*Hyaluronidase Activity* was always determined on the mixture of enzymes and indicator, by means of the spreading reaction in guinea-pig skin and the polysaccharide disaggregation test prescribed by Pirie (1949). At first it was hoped to use Evans blue 1 per cent. in 0.9 per cent. NaCl. as indicator, but this was found to inhibit hyaluronidase action in the polysaccharides disaggregation test. Thus a testis enzyme plus Evans blue solution (1:1v/v) took 94 mins. for the mucopolysaccharide to be disaggregated, whilst the testis enzyme plus 0.9 per cent. NaCl. (1:1v/v) required less than 60 seconds.

*Collagenase from Cl. Welchii* was a gift from Dr. E. Bidwell of the Wellcome Research laboratories. As provided, the enzyme had an activity of 18 Q/ml.

*Nitrogen* was measured by the micro-kjeldahl method.

### Spreading Reactions.

These were of two types:

- (a) Rabbits were anaesthetized with nembutal and the enzyme + indicator solution injected into the stroma of the cornea of one eye, and the control (boiled enzyme + indicator) into the other eye. The blebs were measured after injection, and after the time interval allowed for reaction, and finally (after killing the animal) the latter measurement was confirmed on the excised cornea. With careful injections of 0.05 ml. a circular bleb of initial mean diameter 4 to 6 mm. was consistently produced.

- (b) Isolated ox cornea were placed on squared graph paper covered with filter paper pads soaked in 0.9 per cent. NaCl, and kept at 37°C. The mean initial diameter of the bleb was 8 to 10 mm.

As had been anticipated from the compact structure of the cornea, diffusion was very slow. Accordingly it was decided, in the later experiments, to depart from the conventional pattern of spreading reactions (Bacharach and others, 1940; Humphrey, 1943), and to wait long enough for a significant increase in diameter in the control to have taken place. Measurement was to 0.5 mm., so the significant increase in diameter demanded was 10 per cent. in rabbits and 5 per cent. in ox corneae.

### Results

The activity of the mixture of enzyme and indicator was such that in all cases the time required in the disaggregation tests was less than 60 secs. All enzymes gave a positive spreading reaction in guinea-pig skin. The results set out in Table I and II indicate

TABLE I  
*Rabbit Corneae*

Source of enzyme	No. of animals	Mean % increase in diameter		Time allowed (mins.)
		enzyme	control	
Testis ...	4	0	0	30
	1	0	0	81
	3	14	10	144
Cl. Welchii	2	12	12	160
Pneumococcus	3	14	12	180

TABLE II  
*Ox Corneae*

Source of enzyme	No. of corneae	Mean % increase in diameter		Time allowed (mins.)
		enzyme	control	
Testis ...	5	8	7	130
	4	58	64	1,275
Cl. Welchii	4	52	52	1,275
Streptococcus	3	22	20	360

Diffusion from the bleb produced by injections into the stroma of cornea. Controls contained heat-inactivated enzyme.

no significant difference in the diffusion from the blebs containing enzyme from those acting as controls.

It was considered that if the spreading action of hyaluronidase were very slight it might reveal itself if the compact collagen fibre network could be modified to facilitate diffusion. To that end, two experiments were performed, one involving a swelling of the inter-fibrillar material (Day, 1949), the other destruction of the collagen fibres.

(a) Ox corneae were placed in distilled water for 2 to 3 hours. They became swollen. After injection in the standard manner the mean diameter with both enzyme and control increased by 18 to 20 per cent. in one hour.

(b) The collagenase solution was found to be active in corneal collagen by a modification of the test described by Bidwell and Van Heyningen (1948) under "Estimation of digestion of hide powder". A 60-mesh powder of ox cornea which had been extracted with water and acetone was used as substrate. 400 mg. of this powder were incubated at 37°C., with 10 ml. Palitzsch borate buffer pH 7.09, and 1 ml. collagenase (18 Q/ml.). The control contained heat-inactivated enzyme. After two hours the filtrate from the solution containing the enzyme had 110 per cent. more total nitrogen than had the control solution. The following solutions were made up:

Collagenase	+	streptococcal hyaluronidase	+	Hb	(1:1:1).
Collagenase	+	boiled "	+	Hb	(1:1:1).

Injection into a living rabbit cornea gave a 20 per cent. increase in the mean diameter of the bleb in two hours with each mixture. Comparison of the degree of spreading in both water-swollen and collagenase-treated corneae with that in the untreated cornea indicates a significant increase in the rate of diffusion. Yet even under these conditions hyaluronidase exerted no action as a spreading factor.

### Discussion

It is considered from these experiments that hyaluronidase has no action on the corneal mucopolysaccharides, *in situ*, at least within 24 hours. Accordingly, the suggestion of Mayer and Chaffee (1940) that the observed corneal vascularization following the injection of hyaluronidase was due to the breakdown of the mucopolysaccharides deserves reconsideration.

It is considered that the evidence from spreading reactions alone is insufficient to allow the conclusion that the substrate for hyaluronidase is absent from the cornea. Work is in progress on the fractionation of the mucopolysaccharides of ox cornea. A solution of a material prepared by  $\text{CaCl}_2$  extraction, which, from its analytical figures, could be a mixture of 60 per cent. sulphated mucopolysaccharide and 40 per cent. protein, gave a relative viscosity in buffered saline of 1.6 for a concentration of 0.3 per cent. This viscosity was not reduced by hyaluronidase action, nor did

the solution acquire reducing properties. Full details of these experiments and of the extractions will be given in a further paper.

The presence in pneumococcal filtrates (used by Meyer as a source of hyaluronidase) of other enzymes which attack structures in the cornea would provide a possible explanation of the positive spreading reaction he reports. Presumably the spread of the ulcer produced by pneumococcal infection is effected by enzymatic processes. However, the positive spreading reaction reported to be found with testis enzyme would be difficult to understand.

### Summary

(1) Hyaluronidase from several sources has been found to be inactive as a spreading factor in rabbit and ox cornea.

(2) The suggested action of hyaluronidase in causing vascularization in the cornea by breakdown of the mucopolysaccharides is discussed.

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