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Spreading Factor Activity of Bacterial Chondroitinase.* (24469)

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A number of substances have been shown to increase the spread of intradermally injected indicators; however, "enzymatic spreading factor" has been applied almost exclusively to hyaluronidase(1,2). Many species of bacteria produce hyaluronidase, whose action as a spreading factor in animal tissue has been correlated with depolymerization of hyaluronic acid *in vitro*. Similarly, bacterial chondroitinase has been shown to degrade the polysaccharide chain in chondroitin sulfuric acid *in vitro*(3,4); however, spreading factor activity in animal tissue has not been described. Chondroitin sulfuric acid as well as hyaluronic acid has been isolated from skin of rabbits(5), and more recently from human gingival tissue(6). The present report is concerned with observations on spreading factor activity of chondroitinase produced by oral microaerophilic diphtheroid bacillus.

Materials and methods. All injections were made intradermally in albino rabbits and were given in area approximately 5 cm from spinal column on either side of animal. Bacterial culture supernates or filtrates and testicular hyaluronidase tested for spreading factor activity were administered in 0.5 ml volume consisting of 0.25 ml of test solution and 0.25 ml of 1:2 dilution of india ink in pH 6 buffer(7) and comparing area of spread with that produced by control injection of 0.85% sodium chloride, sterile broth, or test material previously heated at 100°C for 10 minutes. Animals were sacrificed, their skins removed, and areas of spread estimated by measuring diameters on underside of the dermis. To demonstrate increase in permeability of vascular connective tissue, 0.5% Evans blue was administered in 5 ml volume into marginal ear vein 3 minutes prior to intradermal injection of test material. Recordings of accumulation of dye at site of intradermal injection were made 5, 15, and 30 minutes after intradermal injection of test material. Dye accumulations were recorded as area of spread and on an arbitrary visual comparison as to intensity of color. Three strains of a chondroitinase-producing microaerophilic diphtheroid bacillus, designated as strains D2, D3, and D5, isolated originally from human gingival crevices(4), were used. A casitone-yeast extract broth containing 150 mg casitone (Difco), 50 mg yeast extract (Difco), and 50 mg sodium chloride/10 ml of medium was satisfactory for production of chondroitinase by these organisms. The pH of medium was adjusted to 7.2 before sterilization by autoclaving at 120°C for 20 minutes. Inocula were prepared from anerobic cultures maintained on sheep blood agar. Broth cultures were incubated similarly under a mixture of gas containing 95% N_2 and 5% CO_2. All cultures were incubated

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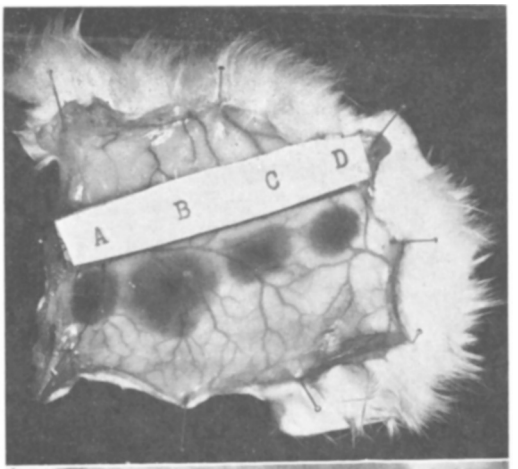

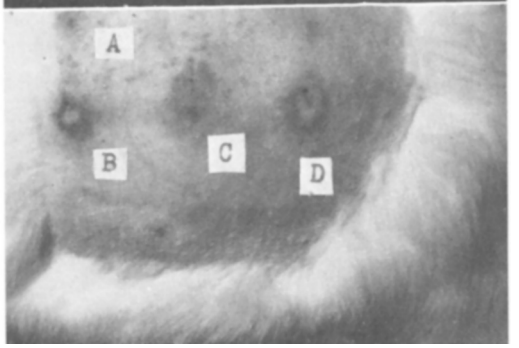

FIG. 1 (top). Spreading of india ink in rabbit skin produced by (A) sterile broth, (B) strain D3 culture supernate, (C) strain D3 heated culture supernate, and (D) 0.85% sodium chloride.

FIG. 2 (bottom). Accumulation of intravenously injected Evans blue in areas of rabbit skin produced by (A) strain D2 heated culture supernate, (B) D2 (C) D3 (D) D5 culture supernates containing chondroitinase activity.

for at least 3 days. Culture filtrates of *Staphylococcus aureus*, strain SAB2, stored at –20°C, were used as source of bacterial hyaluronidase. Testicular hyaluronidase, a product of Armour Lab., was assayed before each experiment. Chondroitinase and hyaluronidase were expressed in turbidity reducing units (TRU) (4,8), using substrates potassium chondroitin sulfate and potassium hyaluronate from human umbilical cord(7).

Results. Fig. 1 demonstrates typical spreading of india ink in rabbit skin by bacterial culture supernate containing 25 TRU/ml of chondroitinase. The marked increase in diffusion of india ink in the area of skin receiving the chondroitinase-containing preparation (Fig. 1B) is to be noted in comparison

TABLE I. Spreading of Intradermally Injected Chondroitinase and Hyaluronidase-Containing Preparations in Rabbit Skin.

Treatment	D2* supernate	D3* supernate	Staphylococcal filtrate†
	Area of spread in cm² (1 hr)		
None	5.9	7.0	3.3
Diluted 1:2 (.85% NaCl)	3.9	5.9	3.1
Diluted 1:5 (.85% NaCl)	2.4	2.4	2.4
100°C for 10 min.	2.4	2.4	2.4

* Signifies 2 strains of diphtheroid bacilli. Supernate contains approximately 25 TRU chondroitinase/ml and < 1 TRU hyaluronidase/ml.

† Filtrate contains approximately 40 TRU hyaluronidase/ml. No chondroitinase activity detectable.

to that produced by any one of the 3 control injections. Table I shows results one hour after administering supernates from 2 strains of diphtheroid bacilli and from a filtrate of *S. aureus*. The chondroitinase-containing preparations effect 2 to 3 times as much spreading factor activity as that produced by staphylococcal filtrate containing 40 TRU of hyaluronidase/ml. Spreading factor activity has apparently disappeared when test materials were diluted 1:5 and as expected when preparations were heated. Heating destroyed also its *in vitro* activity.

Fig. 2 shows accumulation of intravenously injected Evans blue in areas receiving bacterial chondroitinase as compared with accumulation of dye in area receiving a heat-inactivated preparation. Accumulation of some dye in area receiving heat-inactivated preparation (Fig. 2A) can probably be explained on the basis that the medium contains certain nonspecific spreading factors such as peptones shown to increase permeability of connective tissue(9). It may be that chondroitinase in such preparations is not directly responsible for increase in vascular permeability noted. Benditt *et al.*(10) have demonstrated that purification of bull-testis hyaluronidase tends to render it incapable of increasing capillary permeability and that impurities in the extract are actually responsible for the increase in vascular permeability observed. A similar situation may exist with regard to chondroitinase-containing culture supernate. Sub-

stances other than chondroitinase could be responsible for increasing permeability of capillaries and thereby account for accumulation of some Evans blue in areas receiving heat inactivated preparations or sterile broth. The increased area of spread induced by chondroitinase-containing preparation may be due only to depolymerization of ground substance surrounding the capillary bed by the enzyme with increase in vascular permeability due to other, unknown factors. Until one has a highly purified preparation of chondroitinase, it is still questionable that the enzyme is acting directly on the capillary wall. It is certainly conceivable that the enzyme could be acting on components of vascular tissue, since the available substrate may be present(11). Attempts made to dialyze out the interfering substances in the bacterial supernate were unsuccessful.

It was of interest to observe that the action of testicular hyaluronidase and chondroitinase-containing preparations were of the same degree in increasing vascular connective tissue permeability. In both cases after intradermal injection, the bleb produced immediately flattened and accumulation of Evans blue began after about 5 minutes and showed maximum intensity of color after 30 minutes. In contrast, heat-inactivated diphtheroid supernates and heat-inactivated testicular hyaluronidase produced a bleb which remained raised almost throughout the observation period. No accumulation of dye took place in the area receiving heat-inactivated testicular hyaluronidase and only a pale blue accumulation in the area receiving heat-inactivated diphtheroid supernate. If the constituents of the culture supernate, other than chondroitinase, were responsible for the increase in vascular permeability, they are apparently different from those in bull-testis extract since these have been reported to be heat labile(10). This observation has been confirmed in our experiments since heated testicular hyaluronidase had no effect on permeability of vascular connective tissue.

Summary and conclusions. The finding that chondroitin sulfuric acid as well as hyaluronic acid is among the components of human gingival tissue(6), and the demonstration that microorganisms capable of breaking down this substance are present in the gingival crevice, has led to the belief that bacterial chondroitinase may be contributory to the structural breakdown of the gingival elements(4). Experiments here reported demonstrate that bacterial chondroitinase is capable of eliciting the spreading factor phenomenon in connective tissue in the same manner as bacterial and testicular hyaluronidase and may further emphasize the importance of polysaccharases in pathogenesis of periodontal disease.

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