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Occurrence of Hyaluronidase and Lecithinase in Relation to Virulence in *Clostridium welchii.*

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Recent studies of the pathogenesis of the gas-gangrenous infection have focused attention on the role of the enzyme hyaluronidase in influencing the spreading character of the lesion. This enzyme has been isolated from such diverse sources as testicular extract,[1,2] spleen,[3] snake and spider venoms,[4] and extracts of various tumors.[5] It is also produced by many bacteria, including pneumococci[6] and certain strains of non-mucoid hemolytic streptococci,[3,7] and has been demonstrated in cultures of *Cl. welchii, Cl. septicum,* and *Cl. oedematiens.*[8-10] Hyaluronidase has been identified as one of the most active spreading factors;[1,11,12] it acts by hydrolyzing hyaluronic acid, a polysaccharide which is found in the interfibrillar substance of skin,[13] as well as in synovial fluid,[14] vitreous humor,[15] umbilical cords,[15] certain tumors,[5,16,17] and capsules of streptococci of Groups A and C.[18-20]

Although there is widespread acceptance of the thesis that the spreading nature of the *Cl. welchii* infection is due to the presence of hyaluronidase,[11] there are few actual studies of the occurrence of the enzyme in cultures of that organism. McClean and his coworkers[10] found that in 32 strains of *Cl. welchii* which they studied, 12 produced hyaluronidase, and 11 of these were toxicogenic strains, *i.e.* showed lecithinase activity. Of the 20 strains which failed to produce hyaluronidase, 11 were toxicogenic. It is of interest to note that of 20 strains isolated from cases of gas gangrene, 18 produced hyaluronidase.

Since an evaluation of the role of hyaluronidase in the virulence of *Cl. welchii* seemed necessary, the following study was undertaken in order to relate the occurrence of hyaluronidase in freshly isolated strains of *Cl. welchii* to virulence and to the capacity for producing lecithinase. The latter determinations were made on the apparently well-established assumption that lecithinase activity is a measure of concentration of alpha toxin in these organisms.

Methods. Ninety-four strains were isolated, 40 of these from human feces, 24 from soil, and 30 from animal feces. Samples of the source material were heated to 80°C for 10 minutes in skimmed milk containing reduced iron.

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TABLE I.
Hyaluronidase- and Lecithinase-activity, and virulence of strains of *Clostridium welchii*.

Hyaluronidase	Lecithinase	No. Strains	No. Virulent*	No. Toxic†
+	+	32	18	12
—	+	34	16	8
+	—	17	4	10
—	—	11	3	2

*Virulent strains: Those which killed mice with typical local lesions after intramuscular injection of 0.2 ml of broth culture.

†Toxic strains: Those which are not virulent but which killed mice if 0.8 ml of broth culture were given intra-abdominally along with intramuscular injections.

After overnight incubation, tubes showing stormy fermentation were streaked on tryptose agar plates containing 0.025% sodium azide[21] and 5% defibrinated rabbits' or sheep's blood. These were incubated anaerobically for 48 hours at 37°C, after which the typical, well-isolated, hemolytic, mucoid colonies of *Cl. welchii* were picked and transferred to milk containing iron. After overnight incubation, carefully chosen colonies invariably produced typical stormy fermentation. Transfers were then made from the clotted milk to tubes of infusion broth containing cooked meat and, after overnight incubation, these cultures were examined and stored in the refrigerator.

We are not aware of the previous use of sodium azide for the purpose of facilitating the often difficult task of isolating a pure culture of *Cl. welchii*. In media in which the azide was not used, the number of successful isolations was greatly diminished, while plates containing the azide never failed to give well-isolated colonies free of the usual difficulties with spreading contaminants.

For the determinations of hyaluronidase, lecithinase, and mouse virulence, the stock cultures were transferred to another tube of cooked meat medium and also to a tube of plain broth containing 0.25% hyaluronic acid purified from human umbilical cords. The usual difficulties and losses attending the filtration of the highly viscous hyaluronic acid for the purpose of sterilization may be obviated by weighing the dried polysaccharide into a sterile container and allowing the material to soak in 95% ethyl alcohol for a few days. The alcohol may then be carefully evaporated off, and sterile saline or broth added to dissolve the polysaccharide with no loss of material. All tests were performed within a week after the start of the isolation procedure.

Hyaluronidase activity was determined in two ways. The more important and more accurate method was to use the tube containing hyaluronic acid as a substrate. Eighteen-hour cultures in this medium were neutralized to phenol red and centrifugated. The supernate was mixed with acidified horse serum in the presence of acetate buffer at pH 4.2, as in the methods of Seastone,[22] and Kass and Seastone.[23] Since hyaluronic acid usually produces heavy turbidity under these conditions, failure of the turbidity to appear is an indication of hyaluronidase activity. The results could usually be anticipated following the addition of phenol red, since cultures producing hyaluronidase will hydrolyze hyaluronic acid to fermentable end-products with consequent acid production; tubes in which there had been no breakdown showed little change in reaction.

Hyaluronidase was also sought in supernates of centrifugated meat broth cultures by mixing equal amounts of the supernate, dilute hyaluronic acid solution, and phosphate buffer at pH 7.0. These were incubated overnight in the presence of a few drops of toluene and examined by overlaying the mixture on acidified horse serum in precipitin tubes.[22] Since the concentration of hyaluronic acid had previously been adjusted to give a distinct ring when laid over acidified horse serum, failure of any precipitate to appear after incubation with the culture supernate indicated the presence of hyaluronidase in the culture.

[21] Lichstein, H. C., and Soule, M. H., *J. Bact.*, 1944, **47**, 221.

[22] Seastone, C. V., *J. Exp. Med.*, 1943, **77**, 21.

[23] Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.

Lecithinase was detected by a modification of the van Heyningen method:[24] one volume of egg-yolk substrate was mixed with an equal volume of saline and one-half volume of culture supernate. Control tubes were simultaneously set up containing 10-15 units of antitoxin instead of saline. These were read after a few hours' incubation at room temperature.

Mouse virulence was determined by injecting 0.2 ml of the meat broth cultures into the thigh muscles of unprotected mice and also into mice previously protected by the intraabdominal injection of 15 units of antitoxin. The antitoxin which was available to us was active against *Cl. septicum* toxin as well as toward *Cl. welchii* toxin. For this reason, care was taken to use only strains which produced typical mucoid colonies on blood agar and rapid stormy fermentation in milk. Virulent strains produced typical gas-containing lesions and killed unprotected animals within 24 hours. Strains which failed to kill by this procedure were passed through mice by injecting 0.2 ml intramuscularly and 0.8 ml intraabdominally. The heart's blood of animals dying within 24 hours was cultured and passed again through mice by the intramuscular route only. Fourteen strains proved virulent following this procedure, after they had failed to kill in the original test. These are recorded in the data as virulent strains along with those strains which were lethal upon first trial.

The desirability of using freshly isolated strains in studies of this type is illustrated by the observation that certain strains of *Cl. welchii* which were obtained from Dr. Elizabeth McCoy produced hyaluronidase in meat media when tested in 1942 by one of us (E.H.K.) working with Dr. C. V. Seastone, but failed to produce the enzyme when tested recently even when grown in media containing hyaluronic acid. It should also be noted that some of our strains which were originally virulent for mice lost this property after storage in the refrigerator for as little as 6 weeks.

Results. The results, presented in Table I, demonstrate that while the precise role of hyaluronidase in the gas gangrenous lesion must await the completion of histological studies, the enzyme as such is not directly linked with virulence in the strains of *Cl. welchii* studied. Thus, 46% of the virulent strains failed to produce hyaluronidase, whereas only 17% failed to produce lecithinase. Of all the strains examined, the group producing both hyaluronidase and lecithinase showed the greatest number of virulent strains (56%), although almost as many (47%) of the strains in the group producing lecithinase alone were virulent to the mouse.

Evans[25] has recently reported the failure of sera with high anti-hyaluronidase activity to be effective in protecting against infection with *Cl. welchii,* and has pointed out that the protective capacity of antitoxic sera is related to their anti-lecithinase activity.

If a less rigorous test for virulence is applied, namely, the ability of a strain to kill mice after intramuscular injection of 0.2 ml of an 18-hour broth culture along with intraabdominal injection of 0.8 ml of the culture (such strains are "toxic" strains in our designation), it is found that 90% of the hyaluronidase-producing strains and 82% of the lecithinase-producing strains are toxic. We have chosen the more rigorous method as the more significant because we feel that it indicates those strains which are capable of producing an active, typical lesion, rather than a generalized, rapidly occurring toxemia and peritonitis without much local involvement.

While the enzymes occurred in all combinations, including the absence of both, and virulent strains were encountered with each combination, there was no correlation between any combination of enzymes and source of the strain. However, the incidence of lecithinase is considerably greater than that of hyaluronidase; 70% of all strains produced lecithinase, whereas only 52% produced hyaluronidase. The occurrence of virulent strains producing neither enzyme is of great interest, and offers obvious lines for further investigation.

Thirteen of the 14 strains whose virulence was increased by mouse passage produced the same combinations of enzymes before and after such passage. The exception was a strain which produced lecithinase alone before pas-

[24] van Heyningen, W. E., *Biochem. J.*, 1941, **35**, 1246.

[25] Evans, D. G., *J. Path. and Bact.*, 1943, **55**, 427.

sage, but produced hyaluronidase as well after becoming more virulent. While this observation is recorded for its theoretical interest, there is no assurance that the strain recovered after mouse passage was the same strain that was injected, hence the validity of this observation must await confirmation from many sources.

The need for cultivating organisms in the presence of hyaluronic acid in order to demonstrate hyaluronidase is amply borne out by the observation that 49 of 54 strains producing hyaluronidase did so only when grown in media containing hyaluronic acid.

Summary. 1. The isolation of 94 strains of *Cl. welchii* from human feces, soil, and animal feces was facilitated by the use of sodium azide in the culture media. 2. These strains were tested for mouse virulence, and for ability to produce the enzymes lecithinase and hyaluronidase. Fixty-six per cent of the strains producing both enzymes were virulent, as were 47% of those producing only lecithinase. Twenty-four per cent of those strains producing only hyaluronidase and 28% of those producing neither enzyme were virulent. 3. Of 41 mouse virulent strains isolated, 83% produced lecithinase, and 54% produced hyaluronidase. Of all strains isolated, 70% produced lecithinase whereas only 52% produced hyaluronidase.

4. It is concluded that regardless of the role of hyaluronidase in a gangrenous lesion, its *in vitro* production by a given strain of *Cl. welchii* bears no necessary relationship to the virulence of that strain for mice.

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Inactivation of the Antibiotic Activity of Penicillin by Cysteine Hydrochloride. I. Chemical Aspects of Inactivation.

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It is well known that penicillin can be easily inactivated by numerous chemical agents, such as acids, alkalis, etc. Recently Atkinson and Stanley[1] reported the occurrence of "penicidin-suppressors" in a variety of materials, among which they listed thioglycolic acid. They were aware of the fact that the abolition of the antibiotic activity may be the result of either a specific chemical reaction or of an interference with an essential enzyme reaction in a manner similar to the inhibiting effect of p-aminobenzoic acid on sulfonamide bacteriostasis. In another communication,[2] they extended the study to various sulfhydryl compounds including cysteine as well as other reducing agents. Their results suggested to them "that the mechanism of this suppression is a chemical reaction between penicidin and the SH group."

Woodruff and Foster[3] studied the effect of sulfhydryl group (cysteine) on penicillinase, an enzyme which destroys penicillin. In one of their experiments, they used as a control a mixture of penicillin and cysteine with heat-inactivated penicillinase, and found, on incubation, a decrease in the penicillin content. Woodruff and Foster, however, did not extend their investigation of the phenomenon.

In October 1943, during the course of a study on the effect of different chemicals on the stability of penicillin, we observed that penicillin, in contact with neutralized cysteine[4] hydrochloride, rapidly loses its antibiotic activity. In the light of the experiments we are

[1] Atkinson, N., and Stanley, N. F., *Australian J. Exp. Biol. and Med. Science*, 1943, **21**, 249.

[2] Atkinson, N., and Stanley, N. F., *Australian J. Exp. Biol. and Med. Science*, 1943, **21**, 255.

[3] Woodruff, H. B., and Foster, J., *J. Bact.*, (in press).

[4] Cavallito, C. J., and Bailey, J. H., *Science*, 1944, **100**, 390.