STAPHYLOCOCCAL HYALURONIDASE[1]

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Several investigators have been interested in the hyaluronic acid, hyaluronidase system in bacteria and its relation to invasion and virulence. Duran-Reynals (1933) correlated the invasiveness of staphylococci and streptococci with the yield of diffusing factor. Kendall and co-workers (1937) demonstrated hyaluronic acid in the culture media of three types of group A hemolytic streptococci in the mucoid phase, and Seastone (1939) isolated hyaluronic acid from group C hemolytic streptococci in the mucoid phase. Seastone (1943) also showed that 94 per cent of strains from moderate or severe streptococcal infections in man have been found to produce the mucoid polysaccharide in varying amounts. In a group of streptococci from normal throats only about 8 per cent produced hyaluronic acid, all of the producers falling into Lancefield's group A. McClean (1941) demonstrated that capsules and hyaluronidase do not coexist in the same group A or C strain of streptococcus.

McClean (1936) found that hyaluronidase is produced by organisms of the gas gangrene group, and McClean and Hale (1941) showed that the inclusion of potassium hyaluronate in the culture medium of *Clostridium perfringens* resulted in increased production of the enzyme. From this fact he postulated that the presence of hyaluronic acid *in vivo* increases enzyme production, setting up a vicious circle that promotes rapid extension of the infection.

Assay of hyaluronidase. The assay method used was that of Tolksdorf *et al.* (1949), which was kindly made available to us prior to publication. We introduced the following minor modifications:

(1) The potassium hyaluronate was dissolved in 0.1 M acetate-sodium-chloride buffer, pH 6.0, to give the recommended transmittance of 50 ± 5 per cent at a wave length of 600 mμ. A standard curve of turbidity development by hyaluronate and acidified protein showed a transmittance of 47 per cent with a concentration of 0.2 mg per ml. Dilutions of substrate ranging from 0.06 mg per ml to 0.2 mg per ml yielded absorbencies proportional to concentrations of hyaluronate. Therefore, the concentration of 0.2 mg hyaluronate per ml of buffer was used. As the solution ages, the turbidity obtained decreases slightly. The solution is kept in the cold and discarded after 3 weeks. Potassium hyaluronates supplied by the Schering Corporation and by the Wyeth Institute have been used.

(2) Horse serum was used in place of human plasma in the preparation of the acidified protein solution.

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(3) The Coleman junior spectrophotometer was used in the measurement of turbidities.

A search of the literature for references concerning nonenzymic factors that might depolymerize hyaluronate shows that the culture media contain nothing likely to cause false results. Favilli (1940) found that an azoprotein prepared from diazobenzene sulfonic acid coupled with horse serum would reduce the viscosity of synovial fluid. The rate differs from that of hyaluronidase, and pH has very little effect on the reaction. Madinaveitia and Quibell (1941) found that ascorbic acid and certain diazo compounds could cause a fall in the viscosity of hyaluronic acid but that the reaction is independent of pH. Robertson *et al.* (1941) found that ascorbic acid in the presence of H_2O_2 brings about a degradation of synovial mucin; but this is not accompanied by the liberation of reducing sugar.

As a precaution a few "blanks" of sterile media were run for possible turbidity reduction. None showed any reduction.

Production of hyaluronidase. Tryptic digest (pH 7.6) was selected as the culture medium for the following reasons: (1) It has been established (Rogers, 1945) that optimal formation of hyaluronidase occurs only in well-buffered media. (2) Previous studies of staphylococcal coagulase production, made in this laboratory (Walker *et al.*, 1947, 1948), have shown that this medium is highly satisfactory for the growth of the staphylococcus.

Four strains of *Staphylococcus aureus* were assayed quantitatively for hyaluronidase production: L—isolated in April 1946 from a mastoid infection; known to be hemolytic and to produce coagulase in large amounts; Lewis—isolated in April 1948 from a furuncle; known to be hemolytic and to produce coagulase in moderate amounts; 209—Department of Agriculture stock culture used for testing disinfectants; known to be nonhemolytic and a nonproducer of coagulase; and 78—Massachusetts Department of Public Health strain, isolated as a cause of food poisoning; known to be hemolytic and to produce coagulase in small amounts.

One-tenth-ml portions of 18-hour broth subcultures from stock slants were inoculated into 100-ml portions of tryptic digest broth, and the latter was incubated for 1 week at 37 C. The cultures were centrifuged at high speed at 8 C for 45 minutes and the supernatants passed through a Mandler medium filter. The filtrates were assayed by the method cited with the following results: L—hyaluronidase present, the best preparation containing 137 TRU per ml; Lewis—no hyaluronidase; 209—no hyaluronidase; 78—hyaluronidase present, the only 100-ml portion of filtrate assayed showing 40 TRU per ml.

Eleven other strains of *S. aureus* recently isolated from patients, whose histories were unavailable, were assayed qualitatively by the following procedure: Five-ml portions of broth were inoculated from blood agar plate cultures of each strain and incubated at 37 C. The broth supernatant was tested after 24 hours, and again after 48 hours if the 24-hour culture contained no hyaluronidase. Five-tenth-ml portions of the supernatant undiluted, diluted 1:5, and diluted 1:10 with pH 6.0 acetate-chloride buffer were added to 0.5 ml of potassium

hyaluronate solution. Subsequent incubation and turbidity development were followed by the usual quantitative method. The degree of turbidity reduction of each dilution was recorded as: 3 plus—no turbidity; 2 plus—very slight turbidity; 1 plus—moderate turbidity; 0—turbidity equal to that of the usual tube no. 1 in the quantitative method. Of the 11 strains tested, 8 produced no hyaluronidase in 48 hours and 3 produced hyaluronidase in 24 hours.

Schwabacher *et al.* (1945) studied over 800 strains of staphylococci and micrococci. They found that almost 90 per cent of the coagulase-positive group were also positive for hyaluronidase, as demonstrated by the mucin-clot-prevention test. Most of the deficient organisms were isolated from normal carrier sites or apparently healthy wounds. Of 160 coagulase-negative strains none produced hyaluronidase.

In order to obtain some idea of the time when the enzyme was produced, and of its stability in the medium at 37 C, the following was done: One 100-ml portion of medium was inoculated with the L strain, as previously described. At 48, 96, and 144 hours after inoculation, 5-ml samples were withdrawn and centrifuged, and the supernatant was assayed for hyaluronidase. The results were as follows: 48-hour sample—39 TRU per ml; 96-hour sample—45 TRU per ml; 144-hour sample—75 TRU per ml. These values are only approximations because complete removal of the bacteria from the supernatant was not possible, and as a result there was a slight cloudiness not desirable when a spectrophotometric method is used.

It is of interest that at the end of 1 week replicate cultures inoculated with equal amounts of an apparently homogeneous suspension of the seed cultures and grown under the same conditions sometimes show wide variations in their hyaluronidase content. That this could be due to mutant forms that outgrow the normal forms is suggested by the following observations. At one point in the investigation the stock L strain roughened. Coagulase production was negligible and no hyaluronidase was demonstrated. Reversion to the smooth form with simultaneous satisfactory production of coagulase and hyaluronidase was accomplished by growing the organism in blood broth for a week, transfers being made every 24 hours. Duran-Reynals (1933) reported that extracts of R variants of *S. aureus* with rough colonies contained no spreading factor.

Assays of the L supernatant made immediately before and after filtration show that passage through a Mandler medium filter produces no change in hyaluronidase activity.

Duran-Reynals (1939) recommended extraction with 10 ml of water of a 24-hour agar slant culture, subsequent removal of the bacteria, and the determination of spreading factor present in this extract. Haas (1946) mentioned that hyaluronidase appears in the culture medium during growth of the staphylococcus and that it remains in the solution when the organisms are removed. These references raised the question of whether there is any intracellular hyaluronidase in the staphylococcus, and the following procedure was used in an attempt to provide an answer.

The sediment of an L culture whose supernatant showed the presence of

hyaluronidase was drained of the supernatant as completely as possible, washed with saline solution at 5 C, and centrifuged at 5 C for 30 minutes. The supernatant was discarded and the process repeated. The bacterial sediment was resuspended in approximately 15 times its volume of water, covered with an excess of toluene, and incubated at 37 C for 48 hours. The preparation was then centrifuged at 5 C for 45 minutes and the supernatant autolyzate pipetted from beneath the toluene and passed through a Mandler filter. One ml of the filtrate was diluted with 4 ml of 0.1 M acetate, pH 6.0, containing 0.15 M NaCl, and the mixture assayed. No hyaluronidase was present.

The results indicate that, within the limitations of the assay, there is no intracellular hyaluronidase in the staphylococcus.

Attempted partial purification of hyaluronidase. The literature contains few references to methods for purifying bacterial hyaluronidase. Meyer *et al.* (1940) reported on the precipitation of pneumococcic hyaluronidase by sodium flavianate. Rogers (1948) obtained highly active and purified preparations of streptococcal and staphylococcal hyaluronidase. The bacterial culture medium was mixed with kieselguhr and filtered through paper. It was then dialyzed against tap water for 24 hours in the presence of toluene. After adjustment of the dialyzate to pH 5.6, $Fe(OH)_3$ precipitation was employed. After centrifugation in a Sharples supercentrifuge, the precipitate was eluted with 0.2 M Na_2CO_3, as many as five elutions sometimes being necessary. This procedure gives a 20 to 50 per cent yield.

Because of its relative convenience, Meyer's method was attempted in the present study. The bacterial filtrate was adjusted to pH 3.7 with 1 N H_2SO_4 and centrifuged in the cold after 1 hour at 8 C. For each 20 ml of supernatant 1 ml of 4 per cent sodium flavianate (naphthol yellow S) was added. A yellow precipitate immediately resulted. After centrifugation this precipitate was suspended in water, and 0.01 N NaOH was added drop by drop until solution was just complete. The process was twice repeated. Subsequent assay showed very slight activity:

Crude filtrate	*Flavianate preparation*
16.2 TRU per mg nitrogen	5.0 TRU per mg nitrogen

Possibly this low value can be attributed to denaturation of the enzyme by 0.01 N NaOH and to inadequate control of ionic strength. However, assay of the flavianate supernatant showed hyaluronidase present and assay of the pH 3.7 precipitate showed considerable activity. In view of the latter finding isoelectric precipitation was attempted.

Each of six 20-ml portions of a filtrate obtained from a 6-day culture of the L strain was adjusted to a desired pH by the addition of 1 N H_2SO_4. The pH values were determined by the glass electrode. The pH of the original culture filtrate was 7.8. The first 20-ml portion was adjusted to pH 5.5, the second to pH 5.0, the third to pH 4.5, the fourth to pH 4.0, the fifth to pH 3.5, and the sixth to pH 3.0. All portions were stored at 8 C for 1 hour and then centrifuged in the

cold for 15 minutes. The resulting supernatants were clear. Each sediment was drained of its supernatant and dissolved in 20 ml of Na_2CO_3 solution, pH 7.8, which was made by adjusting the pH of 0.1 M Na_2CO_3 with 0.5 M acetic acid to 7.8. The pH of each sediment was checked by the glass electrode and adjusted to 7.8 with 0.5 M acetic acid when necessary. The dissolved sediments were assayed quantitatively; the supernatant, qualitatively. To rule out the possibility that apparent turbidity reduction of the supernatant might be in reality a failure to produce turbidity, caused by increased ionic strength and an unfavorable pH, an equal amount of unincubated supernatant was tested. This method showed turbidity development comparable to that of the upper blank, in the assay. Thus

TABLE 1

Effect of pH on yield and purity of hyaluronidase

pH	ENZYME UNITS/ML	NITROGEN MG/ML	UNITS/MG NITROGEN	QUALITATIVE TEST OF SUPERNATANT 1/10 DILUTION
7.8—original filtrate	106	2.6	41	+++
5.5—sediment	N.S.Q.	(0.002)	—	+++
5.0—sediment	2.4	0.004	600	+++
4.5—sediment	3.1	0.069	45	+++
4.0—sediment	10	0.067	149	+++
3.5—sediment	11	0.078	141	+++
3.0—sediment	35	0.132	265	+++

it is assumed that turbidity reduction, if present, is due to enzyme action. Nitrogen present in the dissolved sediments was determined by the micro-Kjeldahl digestion method of Wong, followed by Koch-McMeekin nesslerization. The results are shown in table 1.

The highest degree of purification associated with significant yield was 6-fold, at pH 3. The double maximum may be real and may offer confirmation of Rogers' (1948) concept of more than one hyaluronidase. An alternative explanation is that it is only apparent, and caused by the coprecipitation of other materials, particularly procoagulase (Walker *et al.*, 1948), in the pH range 4.5 to 3.5.

SUMMARY

Some strains of *Staphylococcus aureus* produce extracellular hyaluronidase when grown in a tryptic digest medium.

The hyaluronidase-positive L strain apparently contains no intracellular hyaluronidase.

The R mutant of the hyaluronidase-positive L strain fails to produce hyal-

uronidase. Hyaluronidase production is resumed upon reversion to the normal S form.

Staphylococcal hyaluronidase can be concentrated and purified to some extent by precipitation at pH 3.0.

The present evidence may indicate the existence of more than one staphylococcal hyaluronidase, as suggested by Rogers (1948).

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