THE PRODUCTION OF HYALURONIDASE BY LANCEFIELD'S GROUP B STREPTOCOCCI[1]

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The relationship of hyaluronidase produced by a microorganism to the virulence and invasiveness of the organism has been widely investigated (Meyer, 1947). The discovery by McClean *et al.* (1943) that *Clostridium perfringens* growing in tissues produced hyaluronidase gave impetus to these investigations. Since, during the course of our studies on bovine mastitis, *Streptococcus agalactiae* (Lancefield's Group B) has been found to be the species of streptococcus most frequently associated with clinical and subclinical mastitis in the area of Madison, the following work was undertaken to determine the extent of hyaluronidase production by these cocci and, also, the relation of the production of hyaluronidase to various environmental factors.

Few studies of hyaluronidase production by the various serological groups of streptococci have been concerned with enzymatic activity by cultures in Group B. McClean (1941) reported production of hyaluronidase by these streptococci; Pierce (1947) found 3 of 11 Group B streptococci isolated from human beings to have activity; Russell and Sherwood (1949) studied a series of cultures from human beings and reported that Group B streptococci exhibited the highest percentage of hyaluronidase producing strains. Sellers (1949) reported that all of 54 strains of Group B streptococci isolated during the course of a study on bovine mastitis produced *in vitro* a low titer of hyaluronidase.

MATERIALS AND METHODS

Cultures. All the bovine cultures used were obtained from blood agar streak plates used to confirm the Hotis test for mastitis streptococci. These tests were conducted by G. R. Spencer and J. Simon of the Department of Veterinary Science as part of their studies of the treatment of mastitis. Each isolate was obtained from one-quarter of the udder of a cow, since previous work had indicated that a single species of streptococcus was usually found in a given quarter. The cultures were picked from the blood agar plates into tryptose broth for purposes of identification and hyaluronidase assay. The cocci were identified on the basis of one or more of the following reactions: (1) their action in hippurate and aesculin (Soulides, 1942); (2) the Lancefield precipitin reaction; (3) the CAMP reaction (Christie, Atkins, and Munch-Peterson, 1944). Cultures which fermented hippurate without the hydrolysis of aesculin, which gave positive precipitin

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reactions with Group B typing serum, or which lysed bovine red blood cells altered by staphylococcal *beta*-toxin were considered to be *S. agalactiae*.

Enzyme assays: substrate. Hyaluronic acid was prepared from the vitreous humor of beef eyes by the method of Seastone (1939) or from human umbilical cords by the method of Tolksdorf, Cassidy, McCready, and McCullagh (1950).

Both preparations were free of protein capable of precipitation by chloroform-butanol mixtures or trichloroacetic acid. Neither preparation formed a mucin clot in the presence of acidified bovine serum but did form a colloidal suspension. The reading on the optical density scale of the Coleman Jr. spectrophotometer was proportional to the concentration of the hyaluronic acid preparation in the serum hyaluronic acid mixture. There was also a direct linearity between the concentration of the hyaluronic acid in a buffer solution and the flow time of the solution in an Ostwald viscosimeter.

Turbidity reaction assay. As a simple method to determine the enzyme activity of the bacterial cultures, the mucin clot prevention test of McClean was employed. One ml of substrate, prepared from 0.25 ml 0.04 per cent hyaluronic acid, 0.5 ml distilled water, and 0.25 ml of bovine serum diluted 1:10 in saline was mixed with 0.5 ml of the supernatant (diluted 1:2 in saline) of an 18 to 24 hr broth culture of the microorganism to be tested and incubated at 37 C for 30 minutes. At the end of the time, the tubes were cooled in ice water and 0.1 ml 2 N acetic acid added to precipitate the remaining hyaluronic acid. Tubes containing sterile broth or broth from inactive cultures became turbid while tubes containing broth from hyaluronidase producing organisms remained clear on addition of the acid. Studies on the characteristics of the enzyme, however, required that the possibility of inhibition by serum be eliminated. For this purpose, the method of Tolksdorf, McCready, McCullagh, and Schwenk (1949) was utilized. One ml reaction mixtures, containing 0.5 ml of substrate (0.04 per cent hyaluronic acid in M/10 buffer), 0.1 or 0.2 ml of broth supernatant from a known active culture, and 0.4 or 0.3 ml of buffer were incubated under the various conditions of time, temperature, pH, or other condition to be tested for effect. Phosphate buffer was usually used. To study the effect of serum inhibitors, acetate buffer was employed, since Haas (1946) has stated that phosphate buffer masks inhibition by normal serum. One-tenth ml of serum diluted 1:10 in acetate buffer and saline was mixed with 0.3 ml enzyme and 0.1 ml buffer and allowed to react for 5 minutes at 37 C before the addition of substrate. Residual hyaluronic acid was precipitated by the addition of 3 ml M/2 acetate buffer, pH 4.2, and 1 ml acidified bovine serum. The acidified serum was prepared by dilution of normal bovine serum 1:10 in M/2 acetate buffer, pH 4.2, adjustment to pH 3.1 with 4 M HCl, and heating in flowing steam for 30 minutes. The turbidities which developed after 30 minutes at room temperature were determined in the Coleman Jr. spectrophotometer to 600 mμ wave length in 2.5 by 15 cm cuvettes. Blanks were prepared by the substitution of buffer for substrate; standard tubes were prepared by substitution of sterile broth for the culture supernatants. Enzymatic activity resulted in a reduction of the final turbidity from the standard.

Viscosity reduction assays. An Ostwald viscosimeter was used to study the vis-

cosity reducing activity of broth culture supernatants of milk samples from cows infected with hyaluronidase producing organisms, and the inhibitory activity for the enzyme of milk and blood serum samples from normal and mastitic cows. The viscosimeter had a 2 ml bulb, a flow time of 63.2 seconds for 5 ml of buffer at 37 C, and a capillary length of 12 cm. The instrument was immersed in a glass jar containing water set in a thermostatically controlled water bath. A 5 ml sample of substrate was introduced into the viscosimeter and readings taken until efflux times within 0.2 seconds on 2 successive readings were obtained. After equilibration of substrate, 0.1 ml of broth culture supernatant of a hyaluronidase producing culture was then added, and the efflux time determined at successive time intervals. Supernatants from haluronidase producing strains reduced the efflux time of the mixture during the course of incubation whereas sterile broth did not reduce the efflux time. Opaque fluids, such as milk, could be tested for hyaluronidase activity by this method. Milk or blood serum samples were examined for antihyaluronidase activity by mixing 0.1 ml of a broth culture supernatant of known viscosity reduction activity with 0.1 ml of the milk or serum in the viscosimeter, incubating the mixture for 5 minutes at 37 C, and adding 5 ml of substrate. The curve of viscosity reduction thus obtained was compared to that of the enzyme substrate mixture alone. The presence of inhibitor was indicated by the failure of the broth culture supernatant to reduce the viscosity of the reaction mixture at the same rate as occurred in the test without the inhibitor. Acetate buffer was used when testing inhibition by milk or serum components, and phosphate buffer was used for other viscosity reduction determinations.

RESULTS

The application of the mucin clot prevention test to a study of 171 strains of streptococci isolated from individual udder quarters disclosed that over 90 per cent of the strains of *S. agalactiae* and *Streptococcus dysgalactiae* produced hyaluronidase *in vitro* (table 1). In contrast, only 17.5 per cent of the streptococci characterized by the hydrolysis of aesculin and comprising such species as *Streptococcus bovis* and *Streptococcus faecalis* were capable of hydrolyzing hyaluronic acid. Fifteen cultures of *beta*-hemolytic Group C streptococci (*Streptococcus zooepidemicus*) did not exhibit hyaluronidase activity. These latter organisms produced a severe mastitis and spread rapidly in the herd in which they were found, in spite of penicillin treatment. They were inhibited *in vitro*, however, by 0.06 units per ml of Penicillin G (Spencer, personal communication). Pierce (1947) reported that 3 of 11 strains of Group B streptococci from human beings had hyaluronidase activity. These were retested by the authors, and the 3 cultures recorded as hyaluronidase positive were again proven positive for the enzyme.

Some characteristics of hyaluronidase from Group B streptococci. Optimum pH. A portion of the umbilical cord hyaluronic acid was dissolved in pH 6.0 M/10 ethylene diamine citrate buffer to a concentration of 0.04 per cent. This solution was mixed with buffer pH values 4, 5, 6, 7, and 8 in a proportion of 1.5 ml substrate to 3.0 ml buffer; 0.5 ml of a 20-hour broth culture supernatant of *S. agalactiae* was added to the mixture at 37 C. The actual pH was then measured by glass

TABLE 1

Hyaluronidase production by streptococci associated with bovine mastitis

SPECIES	LANCEFIELD'S GROUP	HYALURONIDASE ACTIVITY		
		Positive	Negative	Positive
		*numbers**	*numbers**	*per cent*
Streptococcus agalactiae	B	94	8	92.1
Streptococcus dysgalactiae	C	13	1	92.9
Streptococcus zooepidemicus	C	0	15	0
Streptococci	not A, B, or C	7	33	17.5

* Represents numbers of quarters found to contain the species designated.

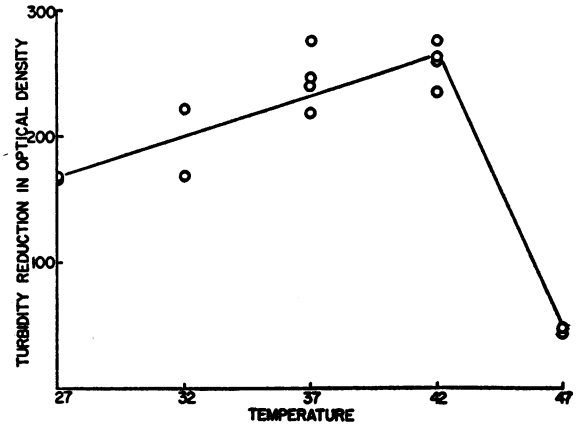

Figure 1. Effect of temperature on turbidity reaction activity of Group B broth culture supernatant fluid. Activity plotted as difference between standard and test turbidities after 30 minutes' incubation at pH 6.0. Units in optical density $\times 10^3$.

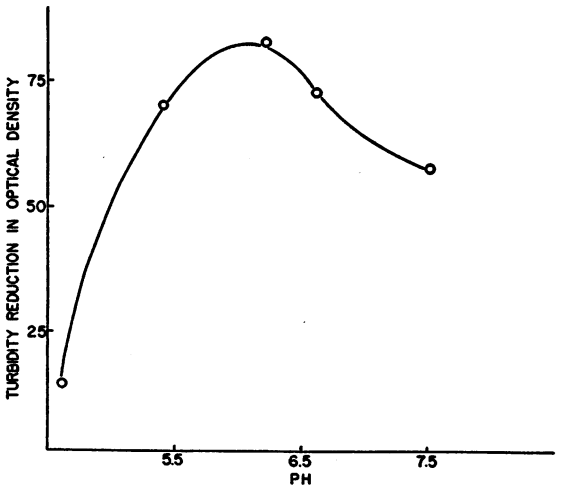

Figure 2. Effect of pH on turbidity reaction activity. Activity as in figure 1, after incubation for 30 minutes at 37 C.

electrode, and the mixture was incubated for 30 minutes. Samples withdrawn at 0, 10, 20, and 30 minutes were precipitated to determine the hydrolysis curve, and the reduction of turbidity from 0 to 30 minutes was compared to the pH. These data are given in figure 1. The values are from the log scale of the photometer and record reduction in turbidity. The pH optimum of the reaction was redetermined by viscosity reduction assay. An aqueous extract of umbilical cord was freed from protein by adsorption on "magnesol" at pH 7.0. The fluid was diluted with equal volumes of M/5 phosphate buffer to give solutions of hyaluronic acid in M/10 buffer at pH values from 5.5 to 8.1 (see figure 2). The viscosities of these solutions were expressed in terms of efflux time of 5 ml substrate in an Ostwald viscosimeter with a flow time of 63.2 seconds at 37 C for the buffer alone. After the substrate had equilibrated, 0.2 ml of a 24-hour broth culture supernatant was added, and the decrease in efflux time after 10 minutes' incubation at 37 C determined. This difference was found to be directly proportional to the concentra-

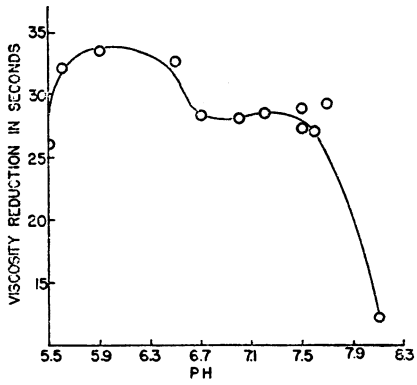

Figure 3. Effect of pH on viscosity reduction activity, plotted as difference between flow times of standard and test solutions after 10 minutes at 37 C. Units, time in seconds.

tion of the enzyme. It is apparent from the figures that the optimum pH for both the viscosity reducing and turbidity reducing activity is in the neighborhood of 6.0, but that activity of the crude enzyme is apparent over a wide range. Two optima are observed for the viscosity reduction activity. This is considered to be evidence that the activity of more than one enzyme was measured by the method.

Temperature. The effect of temperature on the activity of the crude enzyme was determined by the method of Tolksdorf, McCready, McCullagh, and Schwenk (1949). Reaction mixtures, containing 0.5 ml hyaluronic acid (0.04 per cent) in phosphate buffer, 0.3 ml phosphate buffer, and 0.2 ml broth culture supernatant, were incubated at 27, 32, 37, 42, and 47 C for 30 minutes. The turbidity reduction activity was determined according to the procedure of Tolksdorf *et al.*, as described in methods. An apparent optimum temperature of 42 C was obtained for the reaction. Very little activity remained at 47 C. These data are shown in figure 3.

Concentration of substrate. That the enzyme used in these studies was not saturated by the substrate was demonstrated in the following manner: 5, 4, 3, 2, 1,

and 0 ml amounts of 0.04 per cent hyaluronic acid were diluted to 5 ml samples by the addition of the required volume of buffer. The efflux times of each sample were determined, and 0.2 ml of a 20-hour culture supernatant was added. Efflux times were determined at 5 minute intervals for 15 minutes. These data are given in table 2. It was observed that the efflux time for each concentration lessened one-half the difference between the initial flow time and the flow time of the buffer alone at 5 minutes' incubation. It is also apparent that the decrease in efflux time at 5 minutes' incubation time is directly proportional to the original concentration of substrate. Since the initial efflux times of the various concentrations were directly proportional to the concentration, it was assumed that the decrease in viscosity was proportional to the decrease in hyaluronic acid concentration as a result of enzymatic activity. Hence, the enzyme was unsaturated under the conditions of the test. Presumably, the enzyme used in turbidimetric

TABLE 2

*Effect of hyaluronic acid concentration on viscosity reducing in presence of constant amount of enzyme**

HYALURONIC ACID MG/5 ML	INITIAL EFFLUX TIME†	FIVE MINUTE EFFLUX TIME†	TEN MINUTE EFFLUX TIME†	FIFTEEN MINUTE EFFLUX TIME†
2.0	88.6	74.8	70.5	68.8
1.6	83.2	72.0	68.5	67.4
1.2	77.6	70.0	67.0	65.8
0.8	72.2	66.6	65.0	64.8
0.4	66.4	64.0	63.0	62.5
0.0	61.6			

* Enzyme—0.2 ml supernatant from 24-hr broth culture.

† Time in seconds.

pH 6.0; temperature 37 C.

assays was also unsaturated, since greater proportions of broth supernatant were used in that method of assay.

Effect of age of culture and medium for growth. The crude enzyme used for the initial assays was prepared from 18 to 24-hr cultures grown in sugar-free tryptose broth. It was desirable, therefore, to study enzyme production under conditions more nearly approaching the growth of the organism in the cow's udder. Since cows are milked at about 12-hr intervals, and since milk contains abundant carbohydrate, it was necessary to determine the effect of the age of the culture and the inclusion of carbohydrate in the medium on the production of the enzyme. Therefore, 100-ml bottles of tryptose phosphate broth, tryptose phosphate broth plus 0.2 per cent glucose, tryptose phosphate glucose broth plus 1 per cent plasma, sterilized skim milk, and sterilized skim milk plus 2 per cent plasma were prepared. These were inoculated with one ml of a culture of *S. agalactiae* in the maximum stationary phase. Samples were removed at the times indicated in table 3 and tested for enzyme activity, pH, and growth as measured by turbidity, where feasible. The ability of the cultures to produce the enzyme paralleled the period of maximum growth and reached a peak in less than 12 hours. The enzyme

was not destroyed by the acidity produced in the glucose or skim milk media. Viscosity reduction activity was found in milk cultures. The inclusion of plasma did not inhibit the growth and enzyme production of *S. agalactiae* in broth, but had some inhibitory action in milk.

Analysis for hyaluronidase activity of freshly drawn milk. The demonstration of hyaluronidase activity by cultures grown in sterile skim milk led to attempts to demonstrate the enzyme in milk from cows which had been shown to harbor hyaluronidase-producing streptococci. Fresh milk samples were obtained from normal cows, cows shedding Group B streptococci but exhibiting no symptoms, and cows both bacteriologically and clinically affected. Five ml volumes of sub-

TABLE 3

Effect of some environmental factors on the production of streptococcal hyaluronidase (Group B)

AGE OF CULTURE	MEDIA										
	Tryptose phosphate		Tryptose phosphate + 0.2 per cent glucose						Milk		Milk + 2 per cent plasma	
			w/o added plasma			w/plasma						
hours	*pH*	*TR**	*pH*	*T†*	*TR**	*pH*	*T†*	*TR**	*pH*	*VR‡*	*pH*	*VR‡*
2			7.2	0	0	7.2	0	0	6.6	1.8		
3	7.2	0.022										
4			7.2	0	0.004	7.1	0.001	0.007				
6	7.2	0.089	7.1	0	0	7.1	0.015	0.019				
8			7.1	0.014	0.013	6.7	0.058	0.086				
9	7.3	0.135										
10			6.5	0.078	0.016	5.7	0.100	0.136				
12	7.4	0.146	5.3	0.130	0.143	5.2	1.120	0.120				
20									4.8	10.8	5.6	7.8
22			5.2	0.169	0.143	5.2	0.180	0.131				

* Reduction of turbidity from 0 time after 30 minutes' incubation with 0.5 ml substrate, 0.2 ml broth supernatant, and 0.3 ml buffer.

† Turbidity of broth culture measured vs sterile broth blank in units of optical density.

‡ Reduction of efflux time in seconds of 5 ml 0.04 per cent hyaluronic acid plus 0.2 ml milk sample after 9 minutes' incubation at 37 C, pH 6.0.

strate were stabilized in the Ostwald viscosimeter, and 0.2 ml of the milk samples added. Ten different abnormal milk samples from 2 different herds failed to produce any hyaluronidase activity. Four samples from uninfected cows likewise had no activity. The time of incubation was 10 minutes; however, one sample was incubated with the substrate for 47 minutes without any loss in viscosity.

Analysis of milk and blood serum samples for antihyaluronidase activity. One explanation for the lack of activity of the milk samples containing Group B streptococci might be the presence of an inhibitor in the milk. To test this hypothesis, 3 samples of broth culture supernatants of *S. agalactiae* were prepared. One-tenth ml amounts of these preparations were capable of reducing the efflux times of 5 ml of 0.04 per cent hyaluronic acid from between 12 to 19 seconds in 10 minutes' incubation. One-tenth ml of enzyme was mixed in the viscosimeter

with 0.1 ml of a milk sample to be tested for inhibition, incubated at 37 C for 3 minutes, and mixed with 5 ml of substrate in the viscosimeter. The enzyme reaction rate was determined as with the enzyme alone. No significant reduction in the reaction rate occurred with any samples tested. The same method of assay was extended to blood serum obtained from 2 clinically affected cows, an infected heifer free of symptoms, and a bacteriologically noninfected cow. The serums were diluted 1:10 in M/10 acetate buffer, pH 6.0. Definite inhibition of viscosity reduction activity was observed by the serum from the normal cow. Moderate inhibition was affected by the serums from the infected animals. The inhibition was not specific for hyaluronidase from a Group B streptococcus, for it was apparent against an enzyme preparation from a culture of *S. dysgalactiae* (Group C).

DISCUSSION

The optimum pH value obtained by both viscosimetric and turbidimetric procedures agrees with the data given by Rogers (1948) for the enzyme produced by Streptococcus C-7, except that the second peak of activity which he was able to find only by measurement of the release of reducing sugars was visible in the viscosimetric determination. Very little activity was found at pH 4.6 on the turbidity reducing curve.

The rate of increase of turbidity reducing activity with respect to temperature yields a value for Q_{10} of about 1.4; this is in contrast with a value of 2.0 reported by Dorfman (1948) for the testicular enzyme, and of 1.7 for the mucinase of *Clostridium perfringens* by Robertson, Ropes, and Bauer (1940).

The effect of substrate concentration on the rate of reaction indicates that the enzyme under the conditions of the test is unsaturated.

Sellers (1949) discounts the role of hyaluronidase in the pathogenesis of Group B streptococcal mastitis, since he found that organisms isolated by him from various degrees of mastitis exhibited the same low titer of hyaluronidase activity. Our work tends to confirm his belief, since no evidence of hyaluronidase activity could be found in milk samples taken from cows which were carrying the organisms in their udders. This failure could not be ascribed to the presence of an inhibitor in the milk. The inhibitor found in the blood is apparently not carried over into the milk secretion.

The possibility still exists that hyaluronidase may be produced locally *in vivo* and is diluted below the level of sensitivity of the method used. This factor has not yet been explored. Further, the growth of the streptococci *in situ* may be insufficient to produce significant levels of hyaluronidase. Frost and Engelbrecht (1940) found the average milk sample from mastitic cows to contain 10,000 chains of streptococci per ml.

The difference between the percentages of human and bovine strains of Group B streptococci which produce hyaluronidase is of interest. Pomales-Lebron and Morales-Otero (1949) found a marked tendency for bovine strains of Group B streptococci to dissociate toward the rough stage; human strains on the other hand, obtained from other than mammary tissue, were either mucoid or smooth. Our strains, while not characterized on the basis of colony type, were consistently

clumped in broth culture and produced extremely long chains, conforming to criteria established by the preceding authors for rough strains. Pomales-Lebron *et al.* (1947) noted a higher degree of virulence for mice among the human strains as contrasted with the bovine strains. Most studies on the relation of capsule formation to hyaluronidase activity by the streptococci have revealed that the two factors are mutually exclusive (Crowley, 1944; McClean, 1941), although Pike (1948) was able to detect very slight hyaluronidase activity in capsulated strains in the *beta* phase of growth in which the capsules disappeared. It seems possible, then, that a factor exists in most tissues but does not exist in the mammae of the cow which prevents the dissociation of the Group B streptococci *in vivo* to the rough, relatively avirulent, form. Since hyaluronidase production is associated with the rough form, it may be that hyaluronidase formation in the group B streptococci is the result of the environment in which they are most commonly found, rather than a factor in the pathogenesis of the mastitis which they may cause.

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SUMMARY

Over 90 per cent of 112 cultures of *Streptococcus agalactiae* and 14 cultures of *Streptococcus dysgalactiae* produced hyaluronidase when grown *in vitro*. Enzyme activity could be demonstrated for cultures grown with or without added carbohydrate, and was proportional to the growth of the culture. The enzyme activity extended over a wide pH range, was proportional to temperature up to 42 C, and was proportional to the substrate concentration over the range used. No enzymatic activity could be found in milk samples taken from cows infected by hyaluronidase producing streptococci, and this failure was not the result of inhibition by any substance in the milk. An inhibitor was demonstrated in the serum of a normal cow and was effective against hyaluronidase from both *S. agalactiae* and *S. dysgalactiae*. Serum from 3 infected cows had a lower level of inhibition.

REFERENCES

CHRISTIE, R., ATKINS, F. E., AND MUNCH-PETERSON, E. 1944 A note on a lytic phenomenon shown by Group B streptococci. Australian J. Exptl. Biol. Med., **22**, 197–200.

CROWLEY, NUALA 1944 Hyaluronidase production by haemolytic streptococci of human origin. J. Path. Bact., **56**, 27–35.

DORFMAN, A. 1948 The kinetics of the enzymatic hydrolysis of hyaluronic acid. J. Biol. Chem., **172**, 377–387.

FROST, W. D., AND ENGELBRECHT, M. A. 1940 The streptococci. Willdof Book Company, Madison, Wisconsin.

HAAS, E. 1946 On the mechanism of invasion. J. Biol. Chem., **163**, 63–110.

MCCLEAN, D. 1941 The capsulation of streptococci and its relation to diffusion factor (hyaluronidase). J. Path. Bact., **53**, 13–27.

MCCLEAN, D., ROGERS, H. J., WILLIAMS, B. W., AND HALE, C. W. 1943 Early diagnosis of wound infection. Lancet, **1**, 355–360.

MEYER, K. 1947 The biological significance of hyaluronic acid and hyaluronidase. Physiol. Revs., **27**, 335–359.

PIERCE, W. A. 1947 Studies on the hyaluronic acid-hyaluronidase system of hemolytic streptococci. M.S. thesis, University of Wisconsin.

PIKE, R. M. 1948 Streptococcal hyaluronic acid and hyaluronidase. II. Production and subsequent destruction of hyaluronic acid by certain strains of Group A streptococci. J. Infectious Diseases, **83**, 12–18.

POMALES-LEBRON, A., AND MORALES-OTERO, P. 1949 Dissociation among Lancefield's Group "B" streptococci of human and bovine origin. Proc. Soc. Exptl. Biol. Med., **70**, 612–616.

POMALES-LEBRON, A., MORALES-OTERO, P., AND BERAULT, J. 1947 Biological properties and mouse virulence of *Streptococcus agalactiae* and Lancefield's Group "B" streptococci from human sources. Proc. Soc. Exptl. Biol. Med., **64**, 410–412.

ROBERTSON, W., ROPES, MARION W., AND BAUER, W. 1940 Mucinase: a bacterial enzyme which hydrolyzes synovial fluid mucin and other mucins. J. Biol. Chem., **133**, 261–276.

ROGERS, H. J. 1948 Complexity of the hyaluronidases produced by microorganisms. Biochem. J., **42**, 633–640.

RUSSELL, BARBARA, AND SHERWOOD, N. P. 1949 Studies on streptococci. II. The role of hyaluronidase in experimental streptococcal infection. J. Infectious Diseases, **84**, 81–87.

SEASTONE, C. V. 1939 The virulence of Group C streptococci of animal origin. J. Exptl. Med., **70**, 361–378.

SELLERS, K. C. 1949 The production of hyaluronidase by *Streptococcus agalactiae* (Lancefield's Group B). J. Comp. Path., **59**, 109–112.

SOULIDES, D. A. 1942 A note on the use of a composite medium for the determination of *Streptococcus agalactiae*. J. Dairy Sci., **28**, A129 (abstr.).

TOLKSDORF, SIBYLLE, MCCREADY, MARION H., MCCULLAGH, D. R., AND SCHWENK, E. 1949 The turbidimetric assay of hyaluronidase. J. Lab. Clin. Med., **34**, 74–89.

TOLKSDORF, SIBYLLE, CASSIDY, J. W., MCCREADY, MARION H., AND MCCULLAGH, D. R. 1950 *In vitro* studies with hyaluronidase. Ann. N. Y. Acad. Sci., **52**, 1024–1027.