STUDIES ON THE HYALURONIC ACID-HYALURONIDASE SYSTEM OF HEMOLYTIC STREPTOCOCCI

by

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Introduction

The significance of a number of cellular attributes of the hemolytic streptococcus in virulence has been the subject of a great deal of investigation during the past few years. Among other substances, the "M" protein of Lancefield and the capsular polysaccharide hyaluronic acid, have come under suspicion as contributing to the pathogenicity of this species, although in neither case has a complete correlation between the presence of the material in question and virulence been demonstrated.

This paper embodies further work along this line, in which a group of hemolytic streptococcus strains derived from various human pathological conditions have been analyzed with respect to their ability to form the capsular polysaccharide hyaluronic acid, and to elaborate the corresponding hydrolytic enzyme, hyaluronidase.

Review of the Literature

a. The Occurrence and Characteristics of Hyaluronidase

Study of the hyaluronic acid - hyaluronidase system of streptococci actually had its inception in 1929 in connection with investigations of Duran-Reynals (1929) in an entirely different field. In that year this worker called attention to the peculiar property of aqueous extracts of rabbit, guinea-pig, and rat testis of enhancing vaccinal infection in rabbits when these extracts were injected into the skin along with the virus. He was also able to show that the effect was on the host tissue and not on the virus, since (1) the same enhancing effect could be observed when virus was injected into a skin site that had been prepared several days previously by an injection of extract and (2) virus recovered from such lesions showed no alterations in its infectivity.

This substance present in testicular extracts which increased tissue permeability was termed "spreading factor."

Subsequently, spermatazoa were shown by Hoffmann and Duran-Reynals (1931) to contain a principle having spreading properties, and similar substances were found in extracts of malignant tissues (Duran-Reynals and

Stewart, 1931), snake and spider venoms (Duran-Reynals, 1939), and in leeches (Claude, 1937).

Duran-Reynals (1933) found evidence of spreading factor in the culture filtrates of hemolytic streptococci and staphylococci. This property was encountered in most strains of Staphylococcus aureus examined. Fourteen strains of hemolytic streptococcus were studied and classified as "invasive" or "non-invasive" on the basis of virulence tests in rabbits and mice. Spreading factor was assayed by comparing the area of spread in rabbit skin of India ink, injected intradermally both with and without culture filtrates or aqueous cell extracts. Although all of the streptococcus strains were found to produce spreading factor under the conditions of the test, the "invasive" strains were found to produce more of the substance than the "non-invasive."

While very little data is given concerning these 14 strains, three of them are described as of bovine origin, two from cases of erysipelas and the remainder from measles and rheumatic fever, normal throats, and stock strains. While the differences are of such a degree that their significance might well be questioned, it is at least worth noting that the largest areas of spreading were obtained with filtrates of the bovine strains, and human strains derived from cases of erysipelas.

Meyer, Hobby, Chaffee, and Dawson (1940) found

spreading factor in a Group A hemolytic streptococcus isolated from the blood of a fatal case of septicemia. In their method of isolation the cells were dried from the frozen state and ground in the lyophile apparatus. Following this they were suspended in saline, the debris centrifuged, and the spreading factor precipitated from the supernatant with 4% sodium flavianate.

McClean (1914a) studied a number of hemolytic streptococcus strains revived from old stock cultures, with regard to their ability to produce spreading factor. Among the Group A strains included in this study, 4 came originally from cases of scarlet fever, 4 from erysipelas, and 4 from unknown sources.

Prior to animal passage only one of these, a strain from a case of scarlet fever, formed spreading factor. However, after four serial mouse passages, 3 of the 4 erysipelas strains were found capable of producing the factor.

Of 4 Group B strains derived from cases of bovine mastitis, all were found to produce spreading factor without preliminary animal passage.

Of 10 Group C strains derived from various sources, 5 formed spreading factor.

McClean (1936) obtained evidence that spreading factor exists in cultures of pneumococcus and gas gangrene organisms. In connection with these studies he also

established that the spreading principle was antigenic in rabbits. Rabbits were immunized with Cl. welchii toxoid and toxin. It was found that the sera of these rabbits interfered with the activity of spreading factor in rabbit skin.

b. Occurrence and Characteristics of Hyaluronic Acid

Concurrently with the first work on spreading factor, other investigators were studying a substance found in various mammalian tissues and body fluids. This material, a nitrogen-containing polysaccharide, was found to be present in the vitreous humor of cattle by Morner (1894) and was characterized by Meyer and Palmer (1936) as being a polysaccharide acid of high molecular weight, containing a uronic acid, an amino sugar, and possibly a pentose.

In (1936) Meyer and Palmer reported the presence of this material in the vitreous humor of cattle and swine and in Wharton's jelly obtained from human umbilical cords.

Kendall, Heidelberger and Dawson (1937) found the material in Group A hemolytic streptococci by precipitating it from concentrated broth supernatants with 1.25 volumes of alcohol. They described the polysaccharide as consisting of N-acetyl glucosamine and glucuronic acid units and pointed out its probable identity with the polysaccharides of vitreous humor and umbilical cord.

This polysaccharide, called hyaluronic acid, was then encountered in a variety of situations. It was found in bovine and human synovial fluid by Meyer, Smyth and Dawson (1939), in pleural fluid associated with a malignant tumor by Meyer and Chaffee (1939), in Rous sarcoma by Kabat (1939), in rabbit skin by Claude (1940), and in pig skin by Meyer and Chaffee (1941).

It was found to be non-antigenic (Seastone, 1939) in rabbits.

c. The Relation between Hyaluronidase and Hyaluronic Acid

In view of the apparently wide distribution of hyaluronic acid in mammalian skin, and of the fact that spreading factor seemed to exert its activity by increasing the permeability of mammalian skin, it was the logical outgrowth of the two lines of investigation that the action of spreading factor on hyaluronic acid should be determined. In (1939) Chain and Duthie reported the effect of testicular spreading factor on the polysaccharide acids of vitreous humor and synovial fluid. A hydrolysis was found to occur which could be followed by measuring the fall in viscosity of the hyaluronic acid solution undergoing hydrolysis. This fall in viscosity was accompanied by the liberation of reducing substances and N-acetyl glucosamine.

Other "mucinases" were recognized as occurring in various bacterial species, but their relationship to spreading factor went unnoticed for the time being. For example, Meyer, Dubos and Smyth (1936, 1937), and Meyer, Hobby, Chaffee and Dawson (1940) studied a mucinase found in the pneumococcus which was able to hydrolyze polysaccharide acids extracted from bovine vitreous humor, human umbilical cord, Group A hemolytic streptococci, and synovial fluid. However, they were more interested in comparing its properties with those of the pneumococcus autolytic enzyme than in investigating its relationship to spreading factor. Similarly Robertson, Hopes and Bauer (1940) found a mucinase in Cl. welchii capable of hydrolyzing the polysaccharides of vitreous humor, umbilical cord and synovial fluid, but again no study was made of the spreading activity of their preparations, although it was observed that this enzyme would attack a mucin prepared from connective tissue.

However, in (1941), Hobby, Dawson, Meyer and Chaffee carried out a comparative study of the spreading and mucolytic activities of a number of mucinases obtained from clostridia, streptococci, pneumococci, leeches and testes. The following points of similarity between the two activities were observed:

1. If mucolytic activity was present, spreading occurred.

2. Heating at 65 and 100° C. for 30 minutes had an equivalent effect on both spreading and enzyme activity.

1. There was no observable parallelism in the degree of spreading and enzyme activity of any given preparation.

2. All preparations producing spreading did not necessarily contain mucinase.

3. Antisera to mucinase did not inhibit spreading.

On the other hand, McClean (1941c), working with enzymes obtained from bull testis, snake venom and cultures of Cl. welchii, Cl. septicum, staphylococci, pneumococci and hemolytic streptococci, was able to show a 100% correlation between spreading activity and mucolytic activity. The former was measured by noting the difference in bleb size in the skin of rabbits when a hemoglobin solution was injected intradermally both with and without enzyme. Enzymatic activity was measured by following the fall in viscosity in the enzyme-polysaccharide mixtures and testing for the liberation of N-acetyl glucosamine and reducing substances.

In 1936 Meyer and Palmer had observed that hyaluronic acid, when mixed with serum protein, gave a typical mucin clot in the presence of acetic acid. This led to the development by McClean (1943) of the mucin-clot prevention test for estimating mucinase (now called hyaluronidase) activity. The test depends upon the fact that when enzymatic hydrolysis of hyaluronic acid proceeds beyond a certain point, the polysaccharide loses its ability to form the clot with

serum in the presence of acid. In brief, the test is as follows: 0.5 ml. amounts of the desired enzyme dilutions are added to 1.0 ml. amounts of polysaccharide-protein mixture. The tubes are incubated at 37°C. for 20 minutes, after which the tubes are iced and 0.2 ml. of 2N acetic acid added to each. The highest dilution of enzyme preventing clot formation is taken as its M.C.P. titer.

It was also found during this work that antisera prepared in rabbits using purified bacterial spreading factor, would inhibit both skin-diffusing and mucin-clot prevention activity of the homologous preparation. However, it might be pointed out that such a result is not proof that spreading activity and mucolysis are due to the same enzyme. Two enzymes could well have been present in the preparation used for the antigen, two corresponding antibodies therefore developing in the rabbit.

Nevertheless, McClean found that antisera prepared against spreading factors from Cl. welchii and Cl. septicum are species - but not type-specific. Similarly, antisera prepared against hemolytic streptococcus spreading factor were found to be group - but not type-specific. Antisera prepared against enzyme derived from bull testis had no effect against enzymes obtained from mouse testis or from bacteria.

In general it was found, using preparations from 9 sources, that the activity of these preparations was measured

by spreading activity in rabbit skin, ability to prevent the mucin clot and the ability to reduce the viscosity of a polysaccharide solution, was similar in any one preparation, regardless of which of the three methods was used to measure it. The arthor concludes, therefore, that all three tests measure the activity of the same agent.

Favilli (1940) investigated the mucolytic activity of a number of natural and artifical spreading factors. Two natural spreading factors, snake venom (various kinds) and leech extracts, were found to be mucolytic. Of the two artificial spreading factors one, azoprotein (diazobenzene-sulfonic acid coupled to horse serum) was mucolytic, and one, kallikrein (a blood-pressure-lowering substance found in pancreas, salivary gland, and urine)was not.

In (1942) Duran-Reynals reviewed the subject of spreading factors and classified them into two principle groups: 1. Those having both spreading activity, and mucolytic activity in vitro toward hyaluronic acid. 2. Those exhibiting spreading activity only. As examples of this latter group he lists spreading factors found in pig skin, in the whole ground bodies of insects, and the blood-pressure-lowering substance, kallikrein.

c. <u>The Role of Spreading Factor in Bacterial Virulence</u>

It was the natural outgrowth of the finding of

spreading factors associated with bacteria, than an investigation be made of the role these substances might play in the virulence of certain bacterial species.

As already mentioned, Duran-Reynals (1933) found spreading factor in most strains of Staphylococcus aureus which he examined.

Similarly McClean (1936) and Robertson, Ropes and Bauer (1940) found spreading factor associated with gas gangrene organisms. In fact, this association was so constant that McClean, Rogers and Williams (1943) sought to develop a diagnostic test for the presence of gas gangrene organisms based on the detection of the enzyme in edema fluid, using the MCP or viscosity reduction tests.

However, Evans (1943) reported the finding of a number of highly invasive Cl. welchii strains which produced no hyaluronidase.

In a study of 94 strains of Cl. welchii isolated from feces and soil, Kass, Lichstein and Waisbren (1945) could find no correlation between mouse virulence and the ability of a strain to form lecithinase or hyaluronidase.

e. Hyaluronidase Production by the Pneumococcus

The first work done with pneumococcal hyaluronidase was in connection with an attempt (Meyer, Dubos and Smyth;

1936, 1937) to establish its identity or non-identity with the autolytic principle of that organism. This problem was resolved by Meyer, Hobby, Chaffee and Dawson (1940) who found that, following a 49 hour autolysis of pneumococcus cells in the presence of an excess of toluene and the subsequent centrifugation of the cellular debris, the bacteriolytic system could be found chiefly in the sediment, and the carbohydrase system chiefly in the supernatant.

West and Clarke (1938) had noticed a high blood level of glucosamine in cases of pneumonia, a finding which hinted at the possible role of pneumococcal hyaluronidase in the pathology of the disease. However, Humphrey (1944) in a survey of 81 strains of pneumococcus isolated from cases of pneumonia, found no correlation between the amount of hyaluronidase produced by a strain and its clinical virulence.

f. Hyaluronidase Production by Hemolytic Streptococci

Very little work has been done on the problem of a possible correlation between the virulence of hemolytic streptococci and their ability to produce hyaluronidase. As previously mentioned, Duran-Reynals (1933) found spreading factor in 14 strains of streptococcus. However, all of these strains were poorly described and since the work was done prior to the advent of Lancefield's grouping

technique, we do not even have that information on these strains.

Meyer, Hobby, Chaffee and Dawson (1940) found hyaluronidase in a Group A hemolytic streptococcus isolated from a fatal septicemia. This strain was described as "matt." It failed to form mucoid colonies at any time, and was found to be avirulent for mice.

McClean (1941a) encountered enzyme in one scarlatinal and 3 erysipelas strains out of a group of 12 strains of Group A streptococci. He also found it in 4 out of 4 Group B strains of bovine origin, and in 5 out of 10 Group C strains isolated from miscellaneous sources. The possession of a capsule and enzyme production appeared to be mutually exclusive. In fact, encapsulated Group A cells were found to grow without capsules when inoculated into broth containing hyaluronidase.

g. Hyaluronic Acid Production by Hemolytic Streptococci

Seastone (1934) had observed that only young cells - up to 3 hours old - of mucoid streptococcus strains grown in broth were encapsulated and that shortly thereafter the capsules rapidly disappeared. In (1939) Seastone was able to show, in the case of Group C hemolytic streptococci, that this loss of capsule was accompanied by the accumulation of free hyaluronic acid in the culture medium.

McClean (1941a) found that hyaluronidase removed the capsules from 2-hour cells of both Group A and Group C strains in one minute, and postulated that the appearance of small amounts of hyaluronidase in an aging culture might be the reason for the rapid and unexplained loss of capsule by these organisms in broth cultures. Supernatants of 2-hour and 20-hour broth cultures were tested for enzyme activity by means of the MCP test. In 2 out of 3 encapsulated strains, doubtful hyaluronidase activity appeared in the 1-2 dilution of the 20- hour supernatant.

However, in further work McClean (1941b) found that encapsulated Group A streptococci did not develop the capacity to form hyaluronidase after serial passage in broth containing hyaluronic acid.

There is one other report in the literature of the possible coexistence of polysaccharide and enzyme in the same streptococcus strain. It concerns the work of Morison (1941) who treated encapsulated, 3-hour cells with 1% formalin to preserve their capsules. Other young, encapsulated cells were then allowed to lose their capsules for 3 hours in broth at 37°C. The supernatant was neutralized and half of it heated at 56°C. for 2 hours to inactivate any enzyme that might be present. The formalized cells were then seeded into (1) the heated supernatant (2) the unheated supernatant and (3) sterile broth. Loss of capsules

was then followed by the India ink method, the tubes being held at 43, 37, and 20°C. In every case the cells lost their capsules most rapidly in the unheated culture supernatant. If it could be proved that the active principle in the unheated supernatant was hyaluronidase, an example would then be provided of an organism capable of elaborating and dispersing into the surrounding medium not only a polysaccharide, but also the corresponding hydrolytic enzyme of the polysaccharide. As far as the writer is aware, no situation of this kind has as yet been described for pathogenic bacteria.

h. The Role of Hyaluronidase in Streptococcal Virulence

In interpreting the significance of hyaluronidase in streptococcal virulence, McClean points out the advisability of keeping in mind a distinction between the ability of a strain to invade the tissues and set up a more or less localized infection, and true virulence or killing-power. By way of illustration, he performed in vivo tests in mice (1941b) and found that whereas encapsulated strains of Groups A and C exhibited a variable pathogenicity when injected by the intraperitoneal route into mice, they formed only small, circumscribed lesions resembling furuncles when injected intradermally into rabbits. On the other hand, unencapsulated strains of Groups A and C possessing hyaluronidase activity were found to be of very low

virulence when injected intraperitoneally into mice, but produced on intracutaneous injection large areas of necrosis tracking in the direction of gravity in rabbits. The implication of the involvement of such enzyme-producing strains in human erysipelas is obvious. However, outside of the two erysipelas strains reported by Duran-Reynals (1933) and the four reported by McClean (1941a), little work has been done on this relationship. It is worth noting, nevertheless, that five out of these 6 strains were found to be enzyme producers.

Crowley (1944) isolated 376 strains of hemolytic streptococci from various pathological conditions such as scarlet fever, sore throat, puerperal fever, impetigo, burns, and other unspecified skin conditions. A small proportion came from normal throats. All strains were grouped, typed, and examined for encapsulation and hyaluronidase production. Enzyme formation was detected using the MCP test. She reported one Group A strain as coming from a case of erysipelas. It did not form hyaluronidase, and was encapsulated.

Of the 376 strains, 308 belonged to Group A. Sixty-four, or roughly 20% of the Group A strains were enzyme producers, 60% showed capsules, and the remainder formed neither capsules nor enzyme. All Group A enzyme producers belonged to types 4 and 22 and no encapsulated strain could be shown to produce hyaluronidase. No relationship between hyaluronidase production and the

virulence of the strains could be demonstrated. Neither did there seem to be a relationship between enzyme production and the type of inflammatory reaction.

1. The Role of Hyaluronic Acid in Streptococcal Virulence

The significance of the capsular polysaccharide in streptococcal virulence has received much more attention than has the study of the role of the enzyme. Dawson and Olmstead (1934) observed that severe and accute streptococcal infections usually yielded cultures in the mucoid phase, whereas mild, chronic conditions yielded organisms in the smooth phase. Strains which were highly virulent for the mouse invariably were mucoid, although all mucoid strains did not necessarily exhibit mouse virulence.

Seastone (1934) noted that young cells of virulent Streptococcus pyogenes strains were encapsulated during the first 1 or 3 hours of cultural growth and that during the first hour they resisted in vitro phagocytosis in non-immune human blood. When the capsules were lost the cells became susceptible to phagocytosis.

This finding becomes still more significant in view of the work of Morison (1940) who showed that the formation of mucoid colonies by hemolytic streptococci was associated with the presence of capsules.

In (1937) Kendall, Heidelberger and Dawson identified the capsular material of the hemolytic streptococcus with the hyaluronic acid of bovine vitreous humor.

Seastone (1939) was able to correlate the size and persistence of capsules in Group C hemolytic streptococci with the virulence for the guinea-pig, and Hirst (1941) succeeded in protecting mice and guinea-pigs against intraperitoneal infection with Group C organisms by the use of injections of leech extract containing hyaluronidase. However, Hirst could not show a similar protection against Group A strains.

Kass and Seastone (1944) succeeded in protecting mice against Group A infection by the repeated intraperitoneal injection of testicular extracts containing a high titer of hyaluronidase. They also demonstrated increased phagocytosis of organisms in both immune and non-immune blood after treatment of the bacterial cells with hyaluronidase.

Seastone (1945) observed that about 94% of 66 Group A strains obtained from moderate to severe infections formed detectable amounts of hyaluronic acid, whereas only about 8% of 35 strains taken from normal throats and belonging to various serological groups were found to produce the carbohydrate.

Pike (1946) in a similar study arrived at different figures. He found that among 54 Group A strains derived from infections, 48% formed hyaluronic acid. Of 229 Group

A strains from normal throats, 64% formed the acid. The discrepancy between the two figures obtained for normal throats can be partly attributed to the fact that in the one case the test was confined to strains belonging to Group A, while in the other study strains of several groups were included.

The work to be presented in this paper deals for the most part with an examination of the polysaccharide and enzyme-producing capacities of a group of hemolytic streptococcus strains belonging to several serological groups and obtained from cases admitted to the Wisconsin General Hospital and the Infirmary of the Department of Student Health of the University of Wisconsin over a period extending from January, 1946 to April, 1947.* All strains were preserved by lyophilization.

* We are indebted to the Staff of the Wisconsin State Laboratory of Hygiene for its cooperation in obtaining these strains.

Materials and Methods

a. General

Strains were received in the laboratory on blood agar pour plates, usually as part of a mixed flora. Isolated colonies were picked directly to fresh blood agar plates. Throughout the study the blood agar medium used consisted of 1% tryptose (Difco), 0.5% beef extract (Difco), 1.5% agar, 0.5% NaCl, and 5% of defibrinated sheep blood. Subcultures were made from these secondary plates into neopeptone broth for grouping and enzyme studies, and into 10% sheep serum tryptose broth for polysaccharide estimation.

b. Serological Grouping

Cultures were grown for 24 hours in 5 ml. of a medium consisting of 1% neopeptone (Difco), 0.5% beef extract (Difco), 1% dextrose, and 0.5% NaCl. All strains produced fairly abundant growth in this medium. The cultures were grouped using the formamide micro method described by Fuller (1938).

c. Estimation of Mucoid Polysaccharide

The ring-turbidity method of Seastone (1943) was used, with minor modifications, for the measurement of polysaccharide production. Instead of a neopeptone-serum medium, a broth consisting of 2% tryptose (Difco), 1% dextrose, 0.5% NaCl, and 10% sheep serum was used, and in the preparation of the acidified serum reagent, sheep serum was substituted for horse serum. Preliminary studies indicated that acidified serum reagents, prepared with the sera of either of these two animals, gave comparable results.

In this method dilutions ranging from undiluted to 1-80 of the neutralized supernatant from a 24-hour culture in 10% serum broth, are layered over the acidified serum reagent in precipitin tubes. The tubes are allowed to stand for 30 minutes at room temperature after which they are observed under the light from an ordinary reading lamp for the development of a precipitate which forms at the serum-supernatant interface. This precipitate represents the highly insoluble polysaccharide-protein complex which forms between hyaluronic acid and the proteins of the sheep serum, contained both in the supernatant and the serum reagent, at an acid pH. If the tubes are adjusted to neutrality the complex will dissociate, the two components going back into solution.

In the higher dilutions, or lower dilutions if the supernatant contains only a small amount of polysaccharide, a definite ring of precipitate does not form. Instead, the insoluble complex tends to diffuse upward into the supernatant, producing a turbidity. In all cases, the end-point of the polysaccharide titer was taken as the highest dilution of culture supernatant in which this turbidity could be detected.

In order that a fairly high degree of accuracy might be obtained in making these readings, it was found necessary to make certain that the sides of the precipitin tubes were absolutely clean, and that correct lighting conditions prevailed. The light used was an ordinary desk lamp having a metal shade. When readings were made, the tubes were held in such a position, about one inch from the metal shade, that the light passed through them. However, the shade just intercepted the passage of light directly into the eye. This was found to be the optimal angular relationship between tube, light source and eye. Any variation in reading procedure tended to give lower results since faint turbidities were then missed.

No reaction between sterile medium and the acid-serum reagent was observed.

That the reacting substance in the culture supernatants was in reality hyaluronic acid was indicated by the complete, or, in a few cases, nearly complete, loss of

the ability of the supernatants to form either a precipitate or a visible turbidity in the presence of acidified serum, after preliminary treatment with hyaluronidase.

Fifteen supernatants, reacting in titers ranging from 1-20 to 1-80, were treated with an enzyme preparation derived from a rough, type II pneumococcus culture. In all cases, this enzyme was prepared by the method of Dubos (1937). The cells were grown for 24 hours at 37°C. in 5 ml. of the 10% serum tryptose broth described above. The culture was then adjusted to pH 7.0 with N NaOh. An excess of toluol was added and the cells were allowed to autolyze for 24 hours at 37°C. After this the toluol was removed and the tubes centrifuged until clear. One ml. of the fresh autolysate was then added to 1 ml. of culture supernatant and the mixture held at 37°C. for 2 hours. Dilutions ranging from 1-2 to 1-80 were made of this mixture and layered over acidified serum. Autolysate alone gave no reaction with acid serum. In one case it was found that a 1-80 titer had been reduced only to 1-10. In all other cases, however, there was no observable reaction in the tube containing the 1-2 dilution.

Fourteen other culture supernatants, reacting in dilutions ranging from 1-20 to 1-80, were treated in a similar manner, but with a solution of hyaluronidase prepared from bull's testes by the method of Kass and

Seastone (1944)*. No reaction could be observed with the 1-2 dilutions of any of the supernatants treated with this enzyme preparation.

At first an effort was made to detect mixed streptococcal infections, and from the first 18 cultures received four colonies were subcultured. In all cases, the 4 cultures isolated appeared to be identical and no further work was done along this line.

For reproducible results using the ring-turbidity method, it is important to control carefully the cultural history of the strain prior to examination. In all cases, lyophilized cultures were seeded into 2 ml. of 1% serum broth and a loopful of this culture streaked on a blood agar plate. These plates, after 24-hour incubation, were used as the source of inocula for the cultures in 10% serum tryptose broth. The older the blood plate cultures used for the inoculum, the more variable the results. It was also found that lower polysaccharide titers were sometimes obtained if the 10% serum broth tubes were inoculated directly from the 1% serum broth cultures, the blood plate passage being omitted.

In an attempt to arrive at an explanation for the fact that some strains of Group A streptococci produce

* We are indebted to Mr. William Pond for supplying us with this enzyme preparation.

higher polysaccharide titers than others, the observation of Seastone (1939), that Group C encapsulated strains heavily inoculated into broth were encapsulated only during the first 2 or 3 hours of growth, was recalled. He found that most of these strains lost their capsules rapidly after about the fourth or fifth hour and at this time the polysaccharide began to be detectable free in the culture medium. This loss of capsule seemed to correspond, in most cases, with the leveling-off of the rate of growth as the culture entered the maximum stationary phase. It might well be, then, that the amount of capsular material produced by any given strain is a function of the length of time that the strain remains in the logarithmic phase of growth and that this, in turn, is a function of the rate of glycolysis. If this were true, then those cultures producing acid at the most rapid rate would be the poorest polysaccharide producers.

A study was undertaken to determine if such a correlation existed. Eight strains were inoculated into a 5 ml. of 10% serum broth containing brom cresol purple (0.5% indicator in ethyl alcohol added in a ratio of 4 ml. / liter of medium). The inoculum consisted of 0.5 ml. of an 18-hour culture in 1% serum broth. All cultures were shaken at hourly intervals for 8 hours and the shade of the indicator noted. At 24 hours, hyaluronic

acid estimations were done. No correlation between the rate of acid production (free acidity) and the formation of polysaccharide was found. Similarly, no such correlation could be found when the experiment was repeated, using saline-suspension inocula which had been standardized on the photo-electric colorimeter.

In a further attempt to relate polysaccharide production to either the length of time the cultures were in the phase of logarithmic increase or to the total amount of cellular proliferation occurring within the 24-hour incubation period, the growth rates and the final cell concentrations reached by two high-producing and two low-producing strains were determined using turbidimetric measurements.

Twelve-hour cultures of the strains in 5 ml. of 10% serum broth were spun down and resuspended in 10% serum broth to a standard density as determined on the spectrophotometer at a wavelength of 450 gammas. Two ml. amounts of these suspensions were inoculated into 18 ml. of 10% serum broth in 50 ml. Erlenmeyer flasks. Immediately after mixing, 2 ml. samples were withdrawn for turbidity estimations. The cultures were sampled at hourly intervals for 6 hours, and once again at 24 hours.

The 2 ml. samples were spun down in spectrophotometer cuvettes (1.5 ml. minimum capacity), washed once

in saline and resuspended in saline to a volume of 1.5 ml. The turbidities were then estimated on the spectrophotometer at a wave length of 400 gammas. All cultures had nearly reached their maximum densities at the end of 6 hours. There was no great difference in the length of time it took the high producers, as against the low producers, to reach this leveling-off point. However, contrary to expectations, it appeared that the two low-producing strains formed a larger amount of growth in 24 hours than the two high-producing strains. The 24-hour results were checked by total nitrogen determinations using the micro Kjeldahl technique. The 4 cultures were found to contain nitrogen in concentrations roughly proportional to their final optical densities.

This investigation into the dynamics of hyaluronic acid production is of a preliminary nature only, and no attempt has been made as yet to confirm the results obtained. If the results prove to be valid, it will indicate that hyaluronic acid production is not a function of the rate of glycolysis, it does not depend upon the length of time a culture remains in the phase of logarithmic increase, nor upon the total number of cells formed during the 24-hour period. It would seem to be, rather, an intrinsic property of the individual cell.

d. Estimation of Hyaluronidase Production

For the production of Hyaluronidase, the following medium was employed: neopeptone (Difco) 1% beef extract (Difco) 0.5% K_2HPO_4 0.25%, KH_2PO_4 0.05%, and NaCl 0.5%. Rogers (1944) found it advisable to avoid excessive amounts of dextrose in media to be used for hyaluronidase production by streptococci. In the presence of this sugar these organisms rapidly form acidic substances until the pH, after 24 hours, has usually fallen to somewhere in the neighborhood of pH 4.5 - 5.0. Rogers felt that the most logical explanation for the poor production of hyaluronidase by streptococci in the presence of dextrose lay in the fact that, as the reaction of the medium left neutrality and proceeded into the acid range, the cells lost their ability to synthesis the enzyme although they could continue to metabolize and multiply actively.

Of probable significance in this connection is the finding of Dubos (1937), that enzyme solutions held at 37°C. are rapidly inactivated at pH 5.0 and 8.0, whereas such solutions retain their activity for several days at pH 6.5. It is possible that cultures in poorly buffered dextrose broth elaborate as much enzyme as cultures in buffered broth devoid of dextrose, but that in the former case, the enzyme produced is inactivated by the low final pH.

For the determination of the hyaluronidase present in streptococcal cultures, a modification of the quantitative turbidity-reduction method of Kass and Seastone (1944) was employed. This test is based upon the finding that hyaluronic acid, diluted with acetate buffer at pH 4.2, forms a turbidity with acidified serum reagent, and that enzymatic hydrolysis of the polysaccharide lowers or destroys its capacity to form this turbidity.

It was soon realized that for the present studies a quantitative method was not as essential as was a method highly efficient in detecting hyaluronidase production in small amounts. For this reason the quantitative possibilities of the turbidity-reduction method were not utilized. In the method of Kass and Seastone, a suitable dilution of enzyme in pH 6.0 buffer (0.1 M) is allowed to warm for 5 minutes in a 37°C. water bath. "Purified polysaccharide, diluted in the same buffer to a concentration of 0.4 mg/ml. is warmed at the same time and equal amounts of polysaccharide and enzyme solutions are mixed and incubated. At regular intervals 1 ml. samples are removed from the reaction mixture and added to a tube containing 3 ml. acetate buffer (pH 4.2) and 1 ml. acidified serum; after 30 minutes at room temperature, the turbidity is read .....". One turbidity-reducing unit of enzyme activity is defined as "that amount of enzyme which in 30

minutes will reduce the turbidity produced by 0.2 mg. of hyaluronic acid to the equivalent of the turbidity produced by 0.1 mg." The colorimeter readings, made on a Klett-Duboscq photoelectric colorimeter, are converted into milligrams of hyaluronic acid using a reference turbidity curve prepared with known amounts of hyaluronic acid.

In the modification of this method used in the present work, cultures were grown for 24 hours in 5 ml. of the buffered, sugar-free medium previously described. The pH of this medium was found to remain near neutrality throughout the 24-hour incubation period. Following centrifugation, the supernatant was adjusted to about ph 6.0 (the optimum for enzyme activity) using N HCL, and 1 ml. added to 1 ml. of a hyaluronic acid solution containing 0.8 mg./ ml. of polysaccharide in M/15 phosphate buffer at pH 6.0. The final concentration of hyaluronic acid in the mixture was therefore 0.4 mg/ml. Five drops of toluene were added to prevent secondary growth and the mixtures incubated at 37°C. overnight. It was felt that this long incubation period would provide sufficient time for low concentrations of enzyme to effect a detectable amount of polysaccharide hydrolysis. One ml. of the mixture was then diluted with 5 ml. of 0.2M acetate buffer at pH 4.2, and 1 ml. of acidified serum reagent added. After 30 minutes at room temperature

to allow maximum turbidity development, the tubes were read in a Klett-Duboscq colorimeter using one thickness of plate glass as a standard. Any lowering of the turbidity from that produced by 0.4 mg. of hyaluronic acid was considered indicative of enzyme activity. In the presence of 0.4 mg. of hyaluronic acid the depth of the column of fluid necessary to balance the glass plate used on the colorimeter was found to be 2 scale units. Hydrolysis of polysaccharide caused a progressive reduction in the amount of turbidity formed when 1 ml. amounts of polysaccharide-enzyme mixture were exposed to acid-serum reagent. Consequently, deeper columns of fluid were necessary to balance the plate glass standard, resulting in a higher reading on the colorimeter scale. In most cases, enzyme activity completely destroyed the turbidity-forming property of the polysaccharide. In these instances enzyme activity is recorded as greater than 30, which is the maximum scale reading on the colorimeter.

Experimental

A total of 79 strains of hemolytic streptococci were isolated from a wide variety of pathological conditions ranging from Vincent's angina and infectious mononucleosis to scarlet fever, rheumatic fever, and bronchopneumonia. The bulk of the strains came from cases of pharyngitis, "sore throat", and tonsillitis. All strains were grouped and tested for enzyme and polysaccharide production. The results are presented in Table 1.

Table 1

No.	Group**	Polysaccharide	Enzyme***	Case
1.	A	1-80	2	Sore throat
2.	B	-	Over 30*	Bronchopneumonia; sore throat
3.	A	1-40	2	Tonsillitis
4.	A	1-20	3	Tonsillitis
5.	A	1-20	2	Sore throat
6.	A	1-40	2	Scarlet fever
7.	A	1-40	2	Sore throat
8.	A	1-40	2	Sore throat
9.	A	1-20	2	*Tuberculous meningitis
10.	A	1-20	2	Tonsillitis
11.	A	1-20	2	Sore throat
12.	A	1-40	2	Tonsillitis
13.	A	1-80	2	Sore throat
14.	A	1-40	2	Finger infection
15.	A	1-40	2	Tonsillitis
16.	A	1-20	2	Otitis media
17.	B	-	2	Tolsillitis
18.	A	1-80	2	Otitis media
19.	-	-	2	*Infected toe stump
20.	A	1-40	2	Cervical abscess
21.	A	-	Over 30	*Lung abscess or tuberculosis
22.	A	-	Over 30	Tonsillitis

No.	Group**	Polysaccharide	Enzyme***	Case
23.	B	-	7	*Bladder tumor
24.	A	1-80	2	Pharyngitis
25.	B	-	2	*Infectious mononucleosis?
26.	A	1-80	2	Sore throat
27.	A	1-80	2	Otitis media
28.	A	1-40	2	Sore throat
29.	-	-	Over 30	*"tightness of chest"
30.	A	1-20	2	Mastoiditis; otitis media
31.	B	-	8	*Infectious mononucleosis
32.	A	-	Over 30	Purulent rhinitis
33.	A	1-40	5	Sore throat
34.	A	-	Over 30	Acute rheumatic fever
35.	A	1-80	2	*Infectious mononucleosis?
36.	A	1-20	2	Otitis media; brain abscess
37.	A	1-80	2	Endocarditis
38.	C	-	Over 30	*Influenza
39.	A	1-80	2	Sore throat
40.	-	-	Over 30	Tonsillitis
41.	B	-	2	Sore throat
42.	A	-	Over 30	Bronchopneumonia
43.	B	-	2	*Infectious mononucleosis
44.	A	-	Over 30	*Vincent's angina
45.	A	Undil.	2	Acute pharyngitis
46.	A	1-10	2	Tonsillitis
47.	A	1-80	2	*Vincent's angina
48.	A	1-80	2	Sore throat
49.	A	1-20	2	Tonsillitis
50.	A	1-20	2	Tonsillitis
51.	A	1-80	2	Tonsillitis
52.	A	1-40	2	Sore throat
53.	A	1-80	2	Purulent sinusitis
54.	-	-	Over 30	*Infectious mononucleosis?
55.	A	1-20	2	Sore throat
56.	A	1-80	2	Sore throat
57.	A	-	Over 30	Sore throat
58.	A	1-10	2	Sore throat
59.	A	1-40	2	Tonsillitis
60.	-	-	Over 30	*Infectious mononucleosis?

No.	Group**	Polysaccharide	Enzyme***	Case
61.	A	1-40	2	Tonsillitis
62.	A	-	2	*Infectious mononucleosis
63.	A	-	Over 30	Sore throat
64.	A	-	Over 30	*Carrier
65.	A	-	Over 30	Sore throat
66.	A	1-80	2	Tonsillitis
67.	A	-	Over 30	Sore throat
68.	B	-	2	Tonsillitis
69.	A	-	2	Tonsillitis
70.	-	-	Over 30	Pharyngitis
71.	A	1-40	2	Sore throat
72.	A	1-40	2	*Infectious mononucleosis?
73.	B	-	2	Tonsillitis
74.	A	1-80	2	Tonsillitis
75.	A	-	Over 30	Sore throat
76.	A	1-80	2	Tonsillitis
77.	-	-	Over 30	Rhinopharyngitis
78.	A	1-80	2	Tonsillitis
79.	A	1-40	2	Scarlet fever

* - cases of doubtful or non-streptococcal etiology.

** - strains were classified as A, B, C, and "Other groups".

*** - see page 31 for significance of these figures.

When only the cases of probably streptococcal etiology are considered, the total number of strains isolated drops to 61. Of these, 88.5% belonged to Group A, 6.5% to Group B, none to Group C, and 5% to other groups. A breakdown of these strains into groups with their ability to produce hyaluronic acid or hyaluronidase is given in Table II.

Table II

Group	Ability to Produce:					
	Polysaccharide		Enzyme		Neither	
	No. of Strains	%	No. of Strains	%	No. of Strains	%
A	44	81.5	9	16.6	1	1.9
B	0	0	0	0	4	100.0
Other	0	0	3	100.0	0	0

A difficulty encountered in classifying cases into those of streptococcal or other etiology was the frequent occurrence in this series of cases of Vincent's infection and infectious mononucleosis. In both of these conditions, especially infectious mononucleosis, the causative agent has not been determined and as a result, the presence of streptococci associated with them is difficult to evaluate. However, it may be of some significance that in every typical case of infectious mononucleosis encountered in this series, as determined by differential white cell counts, heterophile antibody titer, and general clinical picture, the streptococcus isolated, with one exception, did not belong to Group A. This strain, however, did not produce either hyaluronic acid or hyaluronidase.

The results in Table II indicate that of all Group A streptococcus strains isolated from typical streptococcus infections, roughly 82% were polysaccharide producers and 16% formed enzyme. Thus, nearly all Group A strains isolated

from typical infections were able to form one or the other of these two substances, but in no case was a strain found to be able to produce both. This is in confirmation of the work of McClean (1941a) and Crowley (1944).

The finding that 82% of Group A strains derived from infections form polysaccharide contrasts sharply with the figure reported by Pike (1946) that 48% of the strains he examined were polysaccharide producers. At first it was thought that a possible explanation for this difference might lie in the fact that two different methods were employed for polysaccharide estimation. In Pike's procedure one volume of culture supernatant, adjusted to pH 7.4, was mixed with one volume of acid serum reagent and the amount of turbidity developing after 30 minutes read on the photoelectric colorimeter. This results in the production of a 1-2 dilution of both the supernatant and the acid serum reagent. In the ring-turbidity method of Seastone the adjusted culture supernatant is not mixed with the serum reagent but is layered over it, and any turbidity which develops forms only in the upper supernatant layer. Thus no dilution of the supernatant or of the protein-polysaccharide complex occurs. Theoretically, therefore, it might be expected that the ring-turbidity method would give results about twice as high as the method used by Pike, except for the fact that the readings are made by eye and not on the photoelectric colorimeter.

To test this hypothesis, five Group A strains, producing hyaluronic acid in titers ranging from undiluted to 1-20 were grown in 10% serum broth. Hyaluronic acid estimations were then made on the supernatants in duplicate, using the two methods under consideration. The titer of two of these strains, using the ring-turbidity method, was 1-10. In one case it was 1-20. The supernatants of the other two cultures gave a visible reaction with the serum reagent only when undiluted. In duplicating the method of Pike, equal volumes of supernatant and serum reagent were mixed, allowed to stand at room temperature for 30 minutes, and then read on the Coleman Spectrophotometer in the green band of the spectrum after the machine had been first adjusted to give an optical density reading of zero in the presence of a misture of equal parts of serum reagent and uninoculated medium. In all cases the supernatant-serum reagent mixtures developed sufficient turbidity to cause a definite deflection of the galvanometer, the magnitude of the deflection being roughly proportional to the polysaccharide titer as determined by the ring-turbidity method. It is apparent, that the method which Pike used is as sensitive as that employed in the present study. If, then, the difference in the percentage of polysaccharide producers encountered in these two studies is a valid one, the interesting possibility presents itself that the percentage of enzyme-producing strains in the series

examined by Pike might well have been higher than that found in the present series.

Seastone (1934) observed that young, encapsulated hemolytic streptococcus cells from one-hour old broth cultures are extremely resistant to phagocytosis in non-immune blood. This resistance disappears in about the same time these cells lose their capsules. Older cells are taken up by the phagocytes in large numbers. It was therefore felt that it would be of interest to determine the relative susceptibility of young and old cells of non-capsulated, enzymatic strains to phagocytosis in non-immune blood. Three strains were tested using fresh, defibrinated blood from three non-immune individuals. The cells were grown in 10% serum broth for one hour and 24 hours, spun down, resuspended in saline and exposed to the phagocytes in sealed, rotating tubes at 37° C. for 20 minutes. Smears were then made and stained by Wright's method. The results are given in Table III. The figures represent the number of cells found in 50 phagocytes.

Table III

Strain			Blood Used: A	B	C
48	(young)	E	24	218	158
	(old)		271	110	179
57	(young)	E	82	125	157
	(old)		377	117	437
77	(young)	P	21	117	103
	(old)		469	712	532

First of all it is apparent that the resistance shown by the young cells of the 2 enzymatic strains is not comparable in extent to that found in young cells of encapsulated strains. The behavior of the cells in the presence of the phagocytes of blood A comes nearest to resembling the situation which is encountered with encapsulated strains, but the results obtained with the other two bloods are contradictory. It can be concluded, therefore, that although there seems to be a tendency for the young cells of these strains to be more resistant to engulfment by phagocytes, the effect is not nearly so marked nor as consistent as that observed in encapsulated strains.

If a larger series of phagocytic tests confirms that young cells of enzyme-producing strains are indeed more resistant to phagocytosis in non-immune blood than old cells, the question of why this is so still remains to be answered. In the case of encapsulated strains this phenomenon is not so unique but is a further example of the almost universal principle that in pathogenic bacteria the ability to resist phagocytosis resides in the possession, on the part of the individual cell, of either a well-developed capsule or of the ability to form large amounts of soluble capsular material. However, enzymatic strains do not form detectable amounts of capsular substance and in no case have capsules been observed microscopically on the cells. The only unique substance which these cells excrete seems to be the enzyme, and since testicular hyaluronidase affords protection for young mice against virulent, encapsulated Group A strains, it would seem that the enzyme is incapable of exerting any antiopsonic effect. Furthermore, one would not expect a product of the animal body to exhibit such a property. However, McClean (1943) showed that hyaluronidases from different sources are immunologically specific. Rogers (1946) has demonstrated that the hyaluronidases from Cl. welchii, hemolytic streptococci, and testis all attack the same hyaluronic acid substrate in different ways, with the production of hyrolytic end-products having differing

chemical and physical properties. There is a possibility, then, that while testicular hyaluronidase does not antagonize the action of phagocytes, streptococcal hyaluronidase does, although this seems rather unlikely in view of the rapid inactivation of enzyme which occurs in the animal body (Haas, 1946).

Discussion

Bail (1905) and Rosenow(1907) were the first to show that pathogenic bacteria, growing in animal tissues, are capable of forming substances which, when added to sublethal doses of virulent organisms, cause fatal infections when these doses are injected into test animals. More specifically, Rosenow (1907) found that saline extracts or autolysates of virulent but not avirulent pneumococci contain a substance or group of substances capable of inhibiting the activity of pneumococcal opsins. There is no doubt in the light of later work that virulent organisms produce, not only in vivo but also in vitro, a number of substances which interfere with the defense mechanisms of the animal host. Perhaps the most striking example of a substance of this type is the capsular polysaccharide of the pneumococcus. Felton and Bailey (1936) showed that avirulent type II pneumococci are able to cause fatal

infections in mice if these animals are first given injections of purified type II capsular polysaccharide. Other bacterial products acting against host defense mechanisms are the leucocidins, fibrinolysins, coagulases, and O antigens. In fact, there is no doubt that all toxic products of bacterial cells promote infection by decreasing the resistance of the host in some way.

The hemolytic streptococcus is an outstanding example of an organism that is capable of elaborating a number of these substances, and the determination of the relative significance of these products in the virulence of this species has been the object of much investigation.

The hemolysin which is elaborated by this species has been studied extensively by Todd (1934, 1938a, 1939) who showed that two types are formed. The O hemolysis is oxygen-labile at ordinary temperatures and its activity can be restored by the addition of a suitable reducing agent. The S hemolysin, socalled by virtue of its ease of extraction from streptococcal cells when they are shaken with serum, is sensitive to both heat and acid. Both types are toxic, the S hemolysin causing death by intravascular hemolysis and the O hemolysin by some other means (Todd, 1938b).

In (1905) Ruediger, following the lead of Neisser and Wechsberg (1900), demonstrated that filtrates of broth cultures of virulent hemolytic streptococci have the power

of interfering with the activity of phagocytes toward avirulent streptococcal cells. Channon and McLeod (1929) encountered a substance having anti-phagocytic activity in filtrates of broth cultures of a hemolytic streptococcus isolated from a human knee joint. This substance, considered to be one of that class of agents known as leucocidins, was found to exert a destructive effect on rabbit leucocytes which could be observed microscopically. However, Todd (1938a), using mouse-virulent strains, was unable to find such activity although he employed both the microscopic technique of Valentine (1936), and the technique of Neisser and Wechsberg (1900), in which the amount of leucocytic destruction is determined by measuring the decrease in the ability of washed leucocytes exposed to leucocidin to reduce methylene blue in sealed tubes. It had been found that normal mouse leucocytes, suspended in saline, were clumped by small additions of $\frac{N}{}$NaOH, whereas leucocytes which had been lysed by bacterial filtrates were not. Using this technique, Todd (1942) succeeded in confirming the presence of a leucocidin in hemolytic streptococcus cultures, but this could be shown only if a reducing agent were first incorporated into the culture filtrates. This has led Todd to believe that streptococcal leucocidin is probably identical with streptolysin O.

Tillett and Garner (1933) showed that most pathogenic strains of hemolytic streptococci produce a substance which promotes the lysis of human fibrin clots. This substance is almost invariably present in Group A streptococci derived from human infections (Lancefield and Hare, 1935) and recent evidence has accumulated (Milstone, 1944; Kaplan, Tagnon, Davidson, and Taylor, 1942; Kaplan, 1944) to indicate that fibrinolysin may act as a kinase for certain proteolytic enzymes which are normally present in blood in an inactive state. The possibility that fibrinolysin may be involved in promoting the spread of infection away from a localized focus by dissolving fibrin plugs in adjacent capillaries is of importance.

The work of Dick and Dick (1924a, b, 1925a, b) established that some hemolytic streptococci produce still another toxic substance, the erythrogenic toxin. Injection of this toxin into the skin of susceptible persons usually gives rise to a generalized erythematous rash accompanied by fever and malaise. It differs from streptolysins O and S in being fairly resistant to heat. Foley (1943) showed, on the basis of heat resistance and neutralization tests with specific antitoxin, that this toxin is probably identical with the mouse lethal agent which was described by Harris (1942).

Another substance elaborated by hemolytic streptococci and associated closely with virulence is the "M" protein of Lancefield. This protein seems to be the antigen involved in the type-specific precipitin reaction of Group A streptococci, and antibodies to it are chiefly responsible for the protective action of immune sera. It is found almost exclusively in cultures in the matt and mucoid phases. Although matt strains may be either virulent or avirulent, both types are found to contain about the same amount of "M" protein (Lancefield, 1940-41), indicating that although this protein is always found in virulent streptococcal strains, its presence is not the only essential ingredient in the establishment of the virulent state. That the protein exists at or near the cell surface is suggested by the fact that proteolytic enzymes can destroy the "M" substance of streptococcal cells without affecting their viability (Lancefield, 1943).

The rold of the capsule in streptococcal virulence has been discussed and it appears, from the bulk of the evidence, to be an important one.

On the other hand, the question of whether or not the production of hyaluronidase adds materially to the pathogenicity of a strain remains unanswered. The fact that mice can be protected against infection with encapsulated, virulent, hemolytic streptococci by the repeated

injection of strong hyaluronidase preparations seems to militate against the adoption of the view that hyaluronidase is of any importance. Similarly, the frequent occurrence of hyaluronidase production in Group B, C, and G strains further discourages such a conclusion. The work of Kass, Lichstein, and Waisbren (1945) with Cl. welchii, of Humphrey (1944) with pneumococci, and of Crowley (1944) with the hemolytic streptococcus, also seems to cast doubt on the significance of hyaluronidase in virulence. Crowley, for example, reported that of 308 strains of Group A streptococci isolated from various pathological conditions by a group of public health laboratory units in England, 64 strains, representing about 20% of all Group A isolations, were enzyme producers, but no correlation could be found between enzyme titer and virulence. Although the bulk of these enzymatic strains came from acute infections, there is unfortunately no information as to whether or not they were considered to be the causative agents in these cases. Hence one cannot say that, regardless of the lack of any correlation between enzyme titer and virulence, one is dealing with a group of 64 enzyme-producing hemolytic streptococcus strains, devoid of capsules, which are capable of causing disease.

In the present study, of a total of 61 isolations of Group A streptococci, about 20% were enzyme-producers.

However, the evidence indicated that in only 54 instances could the particular strain isolated by safely regarded as the probable etiological agent in the case. Of this group 9 strains were enzymatic, devoid of capsules, and unable to produce hyaluronic acid in amounts detectable by the test employed. They represent about 16% of the Group A strains included in this group. One of these hyaluronidase-forming strains came from a typical case of tonsillitis. Another was found to be associated with the acute stage of rheumatic fever. A third strain was involved in a case of sore throat and pharyngitis. Still another strain was isolated from the spleen and lung in a fatal case of bronchopneumonia. This case involved a 23-year old pregnant woman who was taken suddenly ill with fever, chills and headache. She became comatose shortly after the onset of symptoms and died about 48 hours later. Although pregnancy was undoubtedly a contributing factor to the fulminating course of this infection, it is still apparent that one is dealing with an invasive organism of considerable virulence.

Five of the enzymatic strains were isolated during an outbreak of mild sore throats in a family group involving the mother and three small children. The outbreak began with the appearance of a rash on the nose and upper lip of one of the children which was diagnosed as impetigo.

This child subsequently became ill with a sore throat. Shortly thereafter the other two children also developed infected throats and from all three an enzymatic Group A streptococcus was isolated. A similar strain was obtained from the throat of the mother who did not become ill. All recovered uneventfully. About a month later one of the children again became sick, with an inflamed throat, enlarged cervical glands and a temperature of 103° F. A throat culture yielded an enzyme-forming Group A strain. A similar strain was recovered at the same time from the throat of one of the other children who remained well.

It seems obvious, then, that Group A streptococcus strains which form enzyme and do not have capsules are capable of causing disease. It remains to be proved, however, that the presence of enzyme has in itself any virulence-enhancing effect. Although not constituting actual proof, statistics tend to lend support to the contention that in order to be virulent, a given strain of Group A streptococcus must be capable of forming either hyaluronic acid or the corresponding enzyme, hyaluronidase. In the present study it was found that, of 54 isolations where it could reasonably be assumed that the organism was the etiological agent, 53 strains, or 98%, were able to produce either the polysaccharide or the enzyme. In only one strain was it impossible to detect

either of these two substances. The correlation of these two properties with virulence in this series is therefore close to 100%.

That the possession of a capsule or the power to form hyaluronidase or "M" protein is not the complete story in streptococcal virulence is apparent. The formation of other toxic cell products such as fibrinolysin must also be taken into consideration. As Dubos has pointed out (1946), whether or not a given strain of bacteria produces an infection or not depends upon the outcome of the interaction between an extremely complex array of forces known compositely as the host defense mechanism on the one hand, and an almost equally complex group of forces known as the bacterial virulence factors on the other. What this outcome will be depends greatly on the physical condition of the host, a factor which varies not only from species to species but also from individual to individual. It also depends upon the condition of the culture employed, such as its age, phase, and certain obscure properties which are in turn dependent upon the medium in which the cells are grown.

The study of virulence in Group A hemolytic streptococci is further complicated by the fact that in the past most tests for pathogenicity have been carried out in mice, using the intraperitoneal route of infection. Whether or not the results so obtained are a true reflection of the

ability of a given strain to produce infection in man by the respiratory route is a question.

What is needed is a comprehensive and quantitative examination, under rigidly controlled conditions, of a group of strains obtained both from cases and carries with regard to their antigenic structure, and their ability to produce hyaluronic acid, hyaluronidase, hemolysins O and S, fibrinolysin, leucocidin, and erythrogenic toxin. A search for a significant difference in the carbohydrate metabolism of virulent and a virulent strains might yield valuable information. Furthermore, a study of the optimum growth temperature, pH, and CO_2 tension of these strains might be useful in the light of the recently proposed theory of Coburn and Pauli (1941) and Coburn (1944) that epidemic strains of hemolytic streptococci are those which are most fully adapted to evironmental conditions existing in the human upper respiratory tract. The value of such a study would depend upon the development of a virulence test in which the experimental animal could be infected by way of the respiratory tract.

Summary

1. In a series of 54 strains of Group A hemolytic streptococci isolated from various pathological conditions such as rheumatic fever, tonsillitis, pharyngitis, otitis media, and bronchopneumonia, 44 strains or about 82%, were found to be hyaluronic acid producers and 9 strains, or about 16% were producers of hyaluronidase.

2. The amount of hyaluronic acid formed did not seem to depend upon the rate of glycolysis in serum-dextrose broth, upon the length of time a culture remained in the logarithmic phase of growth, nor upon the total amount of cells developing in a 24-hour period of growth.

3. Phagocytosis tests indicated that the cells of one-hour cultures of enzymatic, non-capsulated strains were slightly resistant to the action of phagocytes in non-immune human blood, but this resistance was not comparable in extent to that exhibited by young cells of encapsulated strains.

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