A CONTRIBUTION TO THE PHYSIOLOGICAL DIFFERENTIATION OF PNEUMOCOCCUS AND STREPTOCOCCUS, AND TO METHODS OF STAINING CAPSULES.^{1 2}

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GENERAL CONSIDERATIONS.

Pneumococci and streptococci which do not differ in morphology from their classic types can usually be differentiated from each other and identified by their morphological characters without difficulty; but it is equally true that certain cultures of these organisms, either

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at the time of their isolation or after cultivation on artificial media, approach the type of the other so closely that it may be impossible to identify them by their morphology alone. When such morphological variations occur there are no constant and distinctive cultural or pathogenic characters as yet demonstrated, which can with certainty be depended upon as distinguishing marks between these organisms.³

This lack of distinct cultural differences between pneumococci and streptococci has not infrequently led to confusion, but that uncertainty should exist and mistakes be made in identification is not surprising when one considers the characters usually depended upon to distinguish pneumococci from streptococci. Chief among these, as has just been implied, are the morphological features, which are in the case of the pneumococcus a slightly lancet or elongated variation of the more typical coccus form which is characteristic of the streptococci, the arrangement of such cocci in pairs rather than in chains, and the possession of a more or less well-defined capsule. All of these characters are subject to variation or may be absent. Compared with the morphological, the cultural characters are of minor importance, and are variable. They consist in a more watery appearance of the pneumococcus colonies on coagulated blood serum and on agar, and in the usual inability of the freshly isolated pneumococcus to develop readily or at all on gelatin at temperatures below 22° C.

The distinctness of the capsule of the pneumococcus in the body fluids of man and animals, and at times when this organism is artificially cultivated in blood serum, milk or serum agar, has really been depended upon as the chief distinguishing and diagnostic character.

During the past few years, however, from time to time, instances have been reported of distinct capsule formation by organisms which had either been previously identified as streptococcus pyogenes, or

³ On this subject see Welch, *Bulletin of the Johns Hopkins Hospital*, 1892, December, p. 125 et seq.

at the time of their isolation could not be definitely identified by their discoverers as belonging to either this group or to the pneumococci, but were considered intermediate in their character.

Brief Description of Organisms Reported as Capsulated Streptococci.—

Bordet (1) working with an organism previously identified as streptococcus pyogenes described such capsule formation occurring in the peritoneal exudate of infected rabbits.

Schütz's *diplokokkus der Brustseuche der Pferde* (2), Poels and Nolen's streptococcus of contagious pneumonia of cattle (3), and especially the organism described by Bonome (4) as "*streptokokkus der Meningitis cerebro-spinalis epidemica*," may all be looked upon as organisms differentiated on insecure grounds from either pneumococcus or streptococcus. The first two of these organisms, however, are said to be decolorized by Gram's method, and as suggested by Frosch and Kolle (5) in the case of Schütz's organism, may belong to a group intermediate between Fraenkel's diplococcus and the chicken cholera group.

Tavel and Krumbein (6) describe a streptococcus with a capsule, which was isolated from a small abscess on the finger of a child. Capsules were also present in the artificial cultures, and although ordinarily remaining uncolored, could be stained by Loeffler's flagella stain. This organism was said to be differentiated from Fraenkel's diplococcus and also in general from the streptococci (pyogenes) by a rapid and rich growth on gelatin, agar, and potato. A pellicle was formed on broth. The organisms forming this pellicle possessed capsules, but those in the deeper portions of the broth generally lacked the capsule.

In 1897, Binaghi (7) described a capsulated streptococcus isolated from a guinea-pig dead of a spontaneous peribronchitis and multiple pulmonary abscesses. In the pus were found some diplococci and short chains (4 to 6) surrounded by a capsule, which could be made evident by staining with carbol fuchsin. This organism he proposes to call *Streptococcus capsulatus*.

Le Roy des Barres and Weinberg (8) in 1899 published an account of a streptococcus with a capsule. This was isolated from a man who had apparently been infected from a horse which had died of an acute intestinal disorder. The patient neglected the infection and died. Diplococci and short chains furnished with a capsule were found in the subcutaneous tissue at the area of infection. The blood, liver and spleen also contained these organisms. The capsule in all the preparations remained uncolored, but the authors say that its existence was not to be doubted.

Ascitic broth inoculated from the peritoneal exudate of a rabbit dying from the infection gave streptococci in extremely long chains and surrounded by capsules. These were not so distinct as in the case of the organisms in the original smear preparations. Nothing notable was observed in the cultural characters. All fluid media (bouillon, milk and ascitic broth) were said to be strongly acid after twenty-four hours. These authors report that Achard and Marmorek have assured them that they have seen capsulated streptococci, and that Marmorek showed them some preparations in which one of his streptococci presented the same characters as that isolated by them.

Although Le Roy des Barres and Weinberg have used the term encapsulated, they believe that it would perhaps be more prudent to call their organism *streptocoque auréolé*, since they were not able to put this capsule definitely in evidence by staining it.

Howard and Perkins (9) have lately described an organism, probably of the foregoing type, which was present in a tubo-ovarian abscess and in the peritoneal exudate, the blood and some of the organs of a woman dying in the Lakeside Hospital, Cleveland, Ohio. The organisms were biscuit-shaped cocci in pairs, usually arranged in chains of four, six, eight or twenty elements, and surrounded by a wide and sharply staining capsule. In the artificial cultures special capsule stains, it was noted, failed to stain any definite area, but numerous small deeply stained granules were to be seen within the halo, especially near its outer border. Capsules in litmus-milk could be sharply stained. Howard and Perkins propose for the group composed of the streptococci of Bonome, Binaghi, and their own organism, the name *Streptococcus mucosus*.⁴

Reference to the original descriptions of these various capsulated streptococci will show that, with the exception of a rather poorly staining capsule, the majority of these organisms are separated from the typical streptococcus pyogenes or from the pneumococcus by exceedingly slight and unstable morphological and cultural characters. The same is true of the difference observed in their pathogenic action in animals.

There are occasions, then, both within the animal body and in artificial cultivations when it is practically impossible to distinguish definitely between some races of pneumococci and races of strepto-

⁴ Through the kindness of Dr. Perkins, I have had an opportunity of studying the organism of Howard and Perkins. It ferments inulin and in most characters shows a closer affinity with pneumococci, than with true streptococci.

cocci. This difficulty is especially heightened when the pneumococcus has become non-virulent, and at the same time no very typical morphology or capsule formation is to be determined and a tendency to chain formation is marked. Cultures of pneumococci in such condition have come under my notice and were not readily to be distinguished morphologically from streptococcus cultures.

Under these circumstances there has been up to the present time no means of determining morphological or cultural differences by which these organisms could be definitely distinguished. The only recourse is the tedious attempt to revivify the pathogenicity by introduction of the culture into the most susceptible animals and to thus bring again into prominence the lancet-shaped diplococcus type, surrounded by the capsules which characterize the pneumococcus in animal fluids.

My work during the past two years with many cultures of pneumococci and streptococci from various sources has shown certain physiological differences in these organisms, which are, so far as I am able to determine, fixed characters, and have thus far proved unfailing means of distinguishing pneumococci from any streptococcus pyogenes met with. It is, of course, understood that the streptococci here referred to are organisms which would ordinarily be classed as streptococcus pyogenes (*longus*, *brevis*, *conglomeratus*), *erysipelatos*, or *scarlatinae*.

In the course of these experiments I have also been led to the development of simple staining methods for capsules, and to the application of special means, cultural and otherwise, of demonstrating the presence of capsules on both pneumococci and streptococci.

It is the object of the present paper to set forth as briefly as may be these differential and capsule experiments.

THE PHYSIOLOGICAL DIFFERENTIATION OF PNEUMOCOCCUS FROM STREPTOCOCCUS.

PRELIMINARY OBSERVATIONS LEADING TO THE EXPERIMENTS IN DIFFERENTIATION.—My attention was first called to the possibility of a cultural difference between pneumococci and streptococci early in 1900. At

this time I had prepared some serum broth, composed of two parts of broth and one part of an inflammatory exudate from the human pleural cavity. This fluid had originally been rich in cells and was fairly thick, the proteid content being high. Attempts to sterilize at 68° C. had resulted in the solidification or gelatinization of the mixture, and even at 60° C. this was found to take place. The medium was finally sterilized in a fluid condition at 55° C. On March 31, I had occasion to use this medium for the cultivation of a pneumococcus which on March 24 had been isolated from some exudate coming from a case of meningitis in a child following pneumonia. In the serum-broth, after 24 hours' growth at 37° C., the organism brought about a nearly solid coagulum of a yellowish-white color. The culture was examined morphologically by the Welch capsule stain, and was found to be a pure culture of pneumococcus with clearly stained capsules. Subcultures could not be obtained from this tube after 48 hours at 37° C.

The reaction of the uninoculated serum-broth was tested and found to be 0.75% acid, phenolphthalein being the indicator. The coagulated medium containing the pneumococci reacted 2.5% acid. The coagulum dissolved upon the neutralization of the acid.

Shortly subsequent to this, tubes of this same serum-broth were inoculated with two cultures of streptococci. One of the latter was in use in immunizing experiments at the Research Laboratory of the New York Health Department, and the other was isolated by me from the exudate of an empyaema in a child. These, it was noted, did not coagulate the medium even after some days' growth at 37° C.

I was impressed with this difference in coagulative action of the pneumococcus and streptococci in this medium and, realizing that it might indicate more than a temporary difference in the metabolism of these organisms and might thus be of diagnostic value, I made further tests and endeavored to analyze the phenomenon. This, it seemed, might depend upon at least two things—either it might be due to an acid formed by the pneumococcus in the presence of some fermentable substance (probably carbohydrate) not available for the streptococci, or it might be due to an acid produced by the pneumococcus quite independent of a carbohydrate in its nutrient surroundings. That acid was formed and that the precipitate was due in large part to this was indicated by the titration and by the resolving of the coagulum upon the addition of an alkali.

What the fermentable substance was, if there really was one present, which is not unlikely, was not determined.

The normal amount of glucose in fresh beef serum, when the serum is mixed with broth in the proportion of 1 to 2 and the glucose fermented by organisms known to act upon it, such as *B. coli communis* and *B. typhosus*, does not lead to such a coagulation, neither did these organisms give a coagulum when grown in the inflammatory serum broth. It seemed, therefore, that this substance, if it were a carbohydrate, must be one of the less readily fermented, such, for instance, as glycogen; or it might be that some glucoprotein or possibly nucleoprotein, which could be broken up by the pneumococcus, was present in excess of the usual amount. This latter supposition is not unreasonable, as the cell content of the serum was large.

It did not seem probable that glycogen, even if originally present, could long remain in such a serum unchanged by the diastasic and maltasic ferments, which are normally present in blood and such exudates. Experiments were, however, carried on with various carbohydrates, with the hope of thus throwing some light on the problem, and they have led to such interesting and, it is hoped, valuable results, that they form an important part of the present communication.

No attempt has as yet been made to investigate the glucoproteins and nucleoproteins.

CULTURE EXPERIMENTS WITH SERUM-BROTH MEDIA, STERILIZED AT 65°-70° C.—In the first attempt to devise a differential culture medium, fresh beef-serum was diluted with sugar-free broth, reacting 1% acid⁵ to phenolphthalein, in the proportion of two parts of broth to one part of serum. This was then divided into separate portions, one of which was left plain, and to the others were added respectively dextrose 1%, lactose 1%, saccharose 1%, dextrin 1% and starch $\frac{2}{3}$ %.

These media were sterilized at 65°-68° C. for one hour on six consecutive days. They were inoculated with two cultures of pneumococci and four of streptococci.

The dextrose, lactose, saccharose and dextrin media were found to be fermented by the streptococci as well as by the pneumococci, with the formation of a solid, yellowish-white coagulum, due to the resulting acid.

⁵ This broth was neutral to litmus.

The starch medium yielded the most satisfactory differential results. This medium was rapidly fermented by the pneumococci, but was noted only after 13 days' growth at 37° C. as becoming gelatinous from the action of the streptococcus cultures. The plain serum was not coagulated by either organism. Anaërobic cultivations were made. The results were practically the same as given by aërobic growth with the exception of the coagulation of the plain serum-broth by the pneumococcus.

The results indicated by the starch medium were promising. They showed that these starch preparations could be readily fermented by pneumococci with rapid acid formation, and that the streptococci, on the other hand, although they developed in the medium, could not avail themselves of the starch, at least with ease, for it was generally a matter of many days before even a gelatinization of the medium resulted.

As there was, as determined by the iodine test and by the use of Fehling's solution, a marked conversion of the starch during the preparation of the media, and by this the introduction of complicating factors into the tests, changes were made in the mode of preparing the medium.

CULTURE EXPERIMENTS WITH SERUM-WATER MEDIA, STERILIZED AT 65°-70° C.—Distilled water was substituted for the broth in these experiments so as to exclude any hydrolization in the presence of acids and salts, such as are usually present in broth, during the preparation of the starch.

Starch water was prepared by adding 4 grams of powdered starch to 400 cc. of water, boiling for one-half hour, and allowing to stand over night. In the morning a clear fluid could be obtained by pipetting off the water from which the starch particles had settled to the bottom of the flask. This was then added to serum in the proportions of serum 1 part, starch water 2 parts, and sterilized at 68° C. for 1 hour on 6 consecutive days. A medium containing glycogen 1% was also made.

A test of these media showed that in both of them the pneumococcus not only grew readily but induced a coagulum in the glycogen as well as in the starch medium, and this often within 24 hours, at 37° C. The streptococcus cultures grew well, but, as in the first experiment, did not coagulate the starch medium readily, nor bring about coagulation of the glycogen serum, except after many days, and in some cases apparently were not able to do this at all.

Having proved by this preliminary experiment that growth of pneumococci and streptococci would take place in serum diluted with distilled water, it was possible thereafter to avoid the complicating factors incident to the use of broth.

Besides the starch and glycogen media already mentioned, tests were made also with aqueous serum media plus dextrose, lactose, galactose, maltose, saccharose. All of these mono- di- and polysaccharids were readily and rapidly used by the pneumococci, and gave rise in all cases to acid sufficient to cause the coagulation of the albuminous material in the serum. The streptococcus cultures as a rule readily fermented dextrose, galactose, and the maltose (commercial specimen), less readily lactose and saccharose; but starch and glycogen, as noted above, if changed at all, were usually only sufficiently affected by the streptococci to give rise to coagulation after many days and after some evaporation from remaining at 37° C.

These results indicated a marked difference in degree, but not necessarily in kind, between the fermentative power of the pneumococcus and streptococcus cultures as a class. No perfectly distinct differentiation, however, had so far been obtained between the pneumococcus and all of the streptococcus cultures tested.

CULTURE EXPERIMENTS WITH ALKALINE SERUM-WATER MEDIA, STERILIZED AT 100° C.—It was realized that diastasic action going on during the preparation of the media might affect the result of these experiments. Changes might thus be brought about either by the diastase and maltase (glucose) and glycolytic ferments of the

blood or by the action of contaminating bacteria or their accompanying ferments during the long process of low temperature sterilization at 65°-70° C.

The diastasic action of beef serum, as was demonstrated in some experiments, in such mixtures as those used by me, was exceedingly rapid and well marked, as was also the action of maltase. Starch serum mixtures rapidly failed to give the blue reaction with iodine, and after standing for a few hours or even less at the room temperature failed to give even the brown reaction indicating the presence of erythro-dextrin. Those heated to 65°-70° C. failed in these reactions even sooner. This change must be equally true in the case of glycogen, and indicated that our results were not necessarily due to the action of our organism on pure starch or glycogen, but probably on some product of the fermentation of these substances by the enzymes of the blood or by bacteria present during their preparation. Aside from these complications, low temperature sterilizations are at best tedious and unsatisfactory. An attempt was therefore made to eliminate these factors of uncertainty in the preparation of the media.

The first experiment was made by adding small amounts of alkali to the serum-water media, made as above by adding 1 part of beef-serum to 2 parts of distilled water, with the object of converting the proteids into alkali albuminates which could be boiled without coagulating.

By the addition of 1 cc. *n/1* NaOH to the serum water, a perfectly clear fluid resulted which could be sterilized at 100° C., and remain uncoagulated and clear. One-half per cent *n/1* NaOH also gave a non-coagulable medium, but was not quite as clear after boiling as the 1%. Such media are, however, open to the objection that the sugars or starches may be changed when boiled in the presence of an alkali. Gradual reductions of the alkali content were therefore practised and it was eventually found that no added alkali was needed to prevent coagulation in mixtures in the proportion of one part of ox-serum to two parts of distilled water. Such a medium,

although becoming opalescent at boiling temperature, remains perfectly fluid, and is available as a nutrient base for such sugar tests.

Before these last results were obtained, tests were made with the alkaline media plus some of the sugars, and also with the plain alkaline medium. The sugar tests showed nothing especially worth noting except that a longer interval elapsed before the coagulation of the albumins took place, this being due to the additional alkali which had to be neutralized.

The chief point of interest and value developed in connection with the use of a medium without sugar but which had been rendered slightly alkaline by the addition of 0.2% of $n/1$ NaOH. In this medium the pneumococcus cultures invariably gave rise to an opalescence, a gradual whitening and final gelatinization and coagulation. The amount of alkali was afterwards reduced to 0.1% $n/1$ NaOH, and this medium was found to give rise when grown in the incubator at 37° C. to the same opalescence and whitening after about 48 hours, with a firm coagulation after some days, depending upon the culture tested. As may be seen in the appended Table I, no streptococcus cultures bring about visible changes in this medium.

We thus have in this alkaline medium, made of one part of ox-serum and two parts of distilled water plus 0.1% $n/1$ NaOH and sterilized at 100° C., a means of differentiating the pneumococcus from various cultures of streptococci, and so far as my experience goes from all. The behavior of the pneumococcus in this medium seems to indicate a fundamental difference in its metabolism from that of the streptococci. Upon what metabolic process and nutrient ingredients this formation of acid depends has not as yet been determined.

TABLE I.
TEST OF PNEUMOCOCCI AND STREPTOCOCCI IN THE ALKALINE SERUM-WATER MEDIUM. 0.1% *n/1* NaOH.

DAYS.	PNEUMOCOCCUS CULTURES.					STREPTOCOCCUS CULTURES.
	V	VI	VII	VIII	IX	
1	..	..	..	..	..	Twenty-eight cultures were tested; none of these brought about any visible change in the medium.
2	Becoming opalescent.	Becoming opalescent.	..	Becoming opalescent.	..	
3	White.	White.	Becoming opalescent.	White.	..	
4	Gelatinous.	Gelatinous.	White.	Gelatinous.	Becoming opalescent.	
5	Semisolid.	Semisolid.	"	Semisolid.	White	
6	"	"	"	"	"	
7	"	"	"	"	"	
8	+	+	Gelatinous.	+	Gelatinous.	
9	..	..	+	..	Semisolid.	
10	..	..	..	..	+	
20	..	..	..	..	..	

+ Indicates a solid coagulum.

CULTURE EXPERIMENTS WITH SERUM-WATER MEDIA, STERILIZED AT 100° C.—After it had been observed that a mixture of one part of beef-serum and two parts of distilled water could be boiled and thus sterilized without precipitating the albuminous materials present, media were prepared in this manner, containing 1% of various carbohydrates—thus, dextrose, galactose, maltose, lactose, saccharose, dextrin, starch, glycogen, and inulin. These carbohydrates, with the exception of the maltose, which was a fair commercial sample, were of high purity, the galactose, lactose and glycogen⁶ having been especially prepared and tested for this work. Such precautions, it need hardly be remarked, are absolutely essential to the success of these experiments. Samples of glycogen made by manufacturers, and bought in the open market, gave results totally at variance with those obtained when the specially prepared glycogen was tested. More readily fermentable carbohydrates were always found to be present with the glycogen in manufacturers' samples.

This series of experiments with the carbohydrates showed that the monosaccharids — dextrose, levulose, galactose — were fermented readily by practically all streptococci as well as by the pneumococci. This is true also of the disaccharids—maltose, lactose, saccharose—as regards the streptococci as a class, but the development of the coagulum may be much slower than in the case of the monosaccharids, and with certain cultures of streptococci it may be will not occur at all.⁷ In the case of the polysaccharids—dextrin, starch and glycogen—coagulation usually takes place only after many days in streptococcus cultures, and in some cultures apparently does not occur. The non-coagulation is more frequent with glycogen than with starch. Inulin, however, is not fermented by any of the streptococci, although it is readily used by the pneumococci in such serum media. Growing in such an inulin medium, the pneumococcus rapidly gives rise to acid, which leads to the formation of a solid white coagulum which is

These samples of glycogen, lactose and galactose were especially prepared for me by Dr. A. N. Richards, to whom my sincere thanks are due.

⁷ This subject will be discussed more fully in a later paper on the fermentative power of the streptococcus group.

usually complete within 48 hours. By the use of this medium a rapid and constant differentiation can be obtained between pneumococci and the streptococcus pyogenes group.

The differences in behavior of the streptococci in inulin from their behavior in starch and glycogen—the constancy of the non-coagulation—may in part be accounted for by the character of the inulin itself. Inulin is obtained chemically pure and is therefore free from contamination with more readily fermentable carbohydrates. Furthermore, it apparently is not hydrolized during boiling in such a serum medium, nor is it affected by the diastasic ferment present in the blood serum. Glycogen, on the other hand, is only obtained in a comparatively pure state with difficulty, is probably hydrolized to some extent by boiling after it is added to the medium, and is readily acted upon by the diastase of the blood, and subsequently by the maltase, if the preparation be not rapidly carried on or the serum-water heated previous to its addition. These facts may account for some discrepant results, although some are most likely due to differences in action of the streptococci, and may indicate fundamental differences in their physiology.

Preparation of inulin serum-water medium.—The inulin medium is satisfactorily prepared as follows: To one part of fresh, clear beef-serum is added two parts of distilled water, and to this mixture is added 1% of pure inulin. The inulin goes into solution slowly in the cold, and the mixture may be warmed to 55°-60° C. to hasten this solution.⁸ As soon as the inulin is dissolved, the medium should be tubed, and sterilized immediately at 100° C. for ten minutes.⁹ This sterilization is repeated on the two following days. The medium prepared in this way usually becomes only slightly turbid, and no unfavorable changes occur in it.

While this is a favorable method of preparing the inulin medium and no hydrolization or change of the inulin is effected by the action

⁸ The inulin may be dissolved in the distilled water before it is added to the serum.

⁹ Very resistant spores are at times present in inulin. The usual three heatings are then not sufficient, and the medium should be sterilized in the autoclave, at 10 to 15 pounds for 15 minutes.

of the enzymes of the serum, it may also be prepared by first heating the serum-water mixture to 100° C. for some minutes and then adding the inulin. This method should always be followed in preparing other carbohydrate serum-water media. Litmus solution (Merck's highly purified litmus, 5% solution in distilled water) may be added to this media in the proportion of 1%, and changes in reaction thus detected by the color change.

Since sera, even from the same species of animal, differ at times in their initial degree of alkalinity and salt content, specimens are met with, though rarely, which are of so low an alkalinity or have so high a salt content that media made from them are extremely white and opaque, and near the point of heat coagulation. Such media should not be used.

The results of tests of various pneumococcus and streptococcus cultures in 1% inulin serum are shown in Table II. It will be seen that the pneumococcus cultures tested usually coagulated the serum within 48 hours, while the streptococcus cultures, 28 of which were tested, were without effect, although all of them grew at least feebly in the medium.

SUMMARY OF CULTURE EXPERIMENTS.—It is plain from the foregoing experiments that the pneumococcus has a remarkable ability to utilize carbohydrates in its metabolic processes—all mono-, di- and polysaccharids that were tested being rapidly fermented by it as demonstrated by the production of acid. Further than this, the pneumococcus can produce sufficient acid to give rise to a coagulum in a serum medium which is sugar free, or which at least does not contain enough fermentable saccharine matter for the production of appreciable acid by such well-known fermenting organisms as the colon bacillus and the typhoid bacillus, which readily give rise to acid in the presence of glucose.

The streptococci, on the other hand, have apparently more limited and certainly less active fermentative powers. While they usually ferment the monosaccharids and in most instances the disaccharids

TABLE II.
TEST OF PNEUMOCOCCI AND STREPTOCOCCI IN THE INULIN SERUM-WATER MEDIUM.

Days.	PNEUMOCOCCUS CULTURES.								STREPTOCOCCUS CULTURES.
	V	VI	VII	VIII	IX	X	XI	XII	XIII
1	+	+	+	..	..	..	+	+	..
2	..	..	..	+	+	..	..	..	+
3	..	..	..	..	..	Gelatinous.	..	..	..
4	..	..	..	..	..	"	..	..	..
5	..	..	..	..	..	"	..	..	..
6	..	..	..	..	..	"	..	..	..
7	..	..	..	..	..	"	..	..	..
8	..	..	..	..	..	+	..	..	..
9	..	..	..	..	..	..	..	..	..
10	..	..	..	..	..	..	..	..	..
20	..	..	..	..	..	..	..	..	..

+ Indicates a solid coagulum.

Twenty-eight cultures were tested; none of these brought about any visible change in the medium.

rapidly and with ease, the polysaccharids they either do not ferment at all or very slowly. Glycogen and starch are fermented only by certain species or races of the streptococci; inulin is fermented by none so far tested. Furthermore, an appreciable amount of acid is not produced in sugar-free serum media by any streptococcus pyogenes so far as our experience goes.

We may assume, therefore—although one hesitates to dogmatize even after extended experiments on a group of organisms whose physiology is so little understood—that there are distinct differences in respect to the physiological processes of these two forms, or to speak more correctly, of the pneumococcus on one side and the streptococcus group on the other.

The relationship of these organisms may be compared to that which exists between typhoid bacilli and the bacilli of the Gärtner-colon group. The pneumococci resemble the typhoid bacilli in the distinctness and apparent permanence of their cultural characters, while the streptococci seem to form a group which resembles the Gärtner-colon group in presenting grades of fermentative activity, and especially as this activity is displayed by the streptococci in such aqueous serum media as those used in these experiments. In making this comparison it is recognized that the pneumococcus is to be known by positive cultural characters as shown by its ability to ferment the various mono-, di- and polysaccharids, while the typhoid bacilli are chiefly distinguished in comparison with the members of the Gärtner-colon group by the absence of certain physiological activities, such as gas and indol production and the coagulation of milk. Still by this comparison it is simply desired to emphasize the co-extensiveness of the characters, as shown by these experiments, of the organisms commonly recognized as pneumococci, as compared with the variety or variability of the characters displayed by organisms which would ordinarily be classed as streptococci, and probably as streptococcus pyogenes.

EXPERIMENTS ON THE DEMONSTRATION AND STAINING OF CAPSULES
ON PNEUMOCOCCI AND STREPTOCOCCI.

Welch's stain for capsules was the stain constantly used in the earlier of these experiments, and as a rule gave excellent results. Certain conditions, however, at times arose in some of the cultures which interfered with the success of this stain, and I was thus led to seek other methods of staining and of demonstrating the capsules on pneumococci.

NEW METHODS DEVISED FOR STAINING CAPSULES.—After many experiments it was found that a very satisfactory and rapid staining of the pneumococcus capsules could be effected by the following method:

Potassium carbonate method.—This method consists in using as a dye a half-saturated aqueous solution of gentian violet.¹⁰ This is applied for a few seconds to the cover-glass preparation, which has previously been allowed to dry in the air, and has been fixed by heat in the usual manner. No water must be used in diluting and spreading the organisms on the cover-glass. If the organisms come from a solid medium or fluid medium other than fluid sera, some other diluting and spreading fluid such as will be described later must be used. The dye is washed off with a one-fourth per cent (0.25%) aqueous solution of potassium carbonate and the specimen is studied in this fluid. The cover-glass may be sealed on the slide by rimming with vaseline, and evaporation thus prevented. This stain gives remarkable results when used on pneumococci either from fluid or hardened serum media or from the body fluids. (See Figs. 1, 3, 4.) The capsules are large, prominent, and are either stained throughout, or their periphery appears as a dark line or layer, the part next to the deeply stained diplococci being less intensely stained than the periphery. This appearance may be due in part to a precipitation of the dye on the exterior of the capsules by the potassium carbonate.

¹⁰ Gentian violet in substance is added in excess to distilled water and allowed to dissolve to its full extent. The solution is then filtered, and diluted to twice its volume.

Very fair capsule stains may also be obtained by simply staining for about thirty seconds with the ordinary aqueous gentian violet solution (5 cc. sat. alc. sol. to 95 cc. of distilled water) and then washing with a 1% potassium carbonate solution and studying the specimen in the fluid.

Preparations stained by the potassium carbonate method may at times give satisfactory results when dried and mounted in balsam (preferably gum Damar), but this is not the rule.

The following method, however, is eminently satisfactory when it is desired to mount specimens of pneumococci with capsules in balsam.

Copper sulphate method.—In this method a 5% or 10% aqueous solution of gentian violet or fuchsin (5 cc. sat. alc. sol. to 95 cc. distilled water) is placed on the preparation, which has been prepared as previously indicated. The dye is allowed to steam for a few seconds, by gradually passing the cover-glass downwards through the flame several times. After this the staining fluid is washed off with a 20% solution of copper sulphate. This solution may vary from 10% to saturation, but the medium strength, 20%, is generally satisfactory. After washing with copper sulphate, the preparation is dried between filter papers and when thoroughly dry mounted in balsam. The capsules remain of normal size, stained, and distinct. (Figure 2.)

OBSERVATIONS AND EXPERIMENTS ON MEDIA AND CONDITIONS FAVORABLE TO CAPSULE DEVELOPMENT AND STAINING.—The most favorable conditions known for the development of the pneumococcus capsule are found in body fluids of man and animals suffering from an infection with this organism. For instance, capsules may be demonstrated with ease by the usual methods in the blood, serum and inflammatory exudates of the infected rabbit which is, among test animals, one of the most favorable for these experiments. Capsules may be equally well marked in the fresh sputum of pneumonia patients, especially in the early stages of the disease, and in the exudates accompanying such pneumococcus infections as meningitis, otitis media, and empyaema. In sputum and the exudates of these

various localized infections the organisms are, however, frequently degenerated or under chemical conditions unfavorable for capsule staining, and satisfactory results are then not easily to be obtained. The same is true of the scrapings from lungs of patients dead of pneumonia, often even in the stage of red hepatization. Under these conditions a longer exposure to the staining reagent is necessary, before the organisms and capsules are brought into prominence, and even then the results in nowise compare to those obtained with organisms in fresh sputum or the body fluids of such an animal as the rabbit.

It was shown by Ortman (10), as early as 1888, that outside of the animal or human body pneumococci regularly developed capsules when cultivated in blood serum. Welch¹¹ in 1892 and Paulsen (11) in 1893 called attention to the development of capsules on these organisms in milk, and Schmidt (12) showed that sputum media favored the formation or preservation of pneumococcus capsules. Schabad (13) made a similar observation in 1896 in regard to blood agar. Frosch and Kolle (14) refer to the demonstration of capsules on pneumococci cultivated in Guarnieri's medium, and rather indefinite statements in regard to capsules on pneumococci coming from broth and other cultivations may be found in various articles on the pneumococci.

In my own work, most of these experiments have been repeated and the results confirmed. In the serum media of various composition used during the experiments on the physiological differentiation of pneumococci and streptococci, the pneumococci not only grew readily but developed distinct and well-marked capsules. This capsule formation seems to be independent of the length of time the organism has been under artificial cultivation. One of the most favorable media of all for the development of capsules was that made of 1% starch-bouillon and serum—serum 1 part, bouillon plus 1% starch 2 parts—and sterilized at 65°-70° C. In this medium, according to my experience, capsules are developed with great regu-

¹¹ loc. cit.

larity and may be stained without difficulty. The same is true of pneumococci grown on Loeffler's coagulated blood serum, or on coagulated serum without glucose; and these organisms may be prepared for staining by simply spreading them on the cover-glass in some of the condensation water from the serum tube. This use of condensation water may also be successful in the case of organisms growing on plain or glycerine agar, but the most successful method to preserve or perhaps to accentuate the capsule on pneumococci or streptococci coming from artificial media, other than fluid serum media, is to use a drop of serum as the diluting and spreading fluid for the cover-glass preparations. Some of the carbohydrate serum mixtures, such as the starch or glycogen media and the alkaline media, often serve this purpose better than unmodified or fresh serum.

My first experiments in this direction were with the serum starch mixtures used for cultural purposes. It occurred to me that such a fluid in which the pneumococcus capsules were always present and demonstrable by staining might serve to preserve or even develop them on organisms when these were transferred to a drop of the fluid from various artificial media, such as broth, agar, etc. This is successful with pneumococcus nearly without exception, and the presence of a capsule bears little relation to the time the organism has been cultivated artificially, or the medium from which it comes. It is well, however, to have fresh cultures, say usually not over 24 hours old.

It is to be noted that such a fixing medium does not bring the capsule into prominence by simply serving as a deeply stained background and leaving unstained around the organisms the so-called "retraction zone." By the potassium carbonate method the diluting medium often remains practically unstained, while the capsule stands out plainly, either stained definitely throughout, or with a distinct peripheral line or layer which shows to perfection when the organism is free from surrounding detritus. In the copper sulphate method the capsules stain uniformly while the field may or may not be free from detritus.

In smears from the serum of infected animals, *i. e.*, rabbits with pneumococcus, the potassium carbonate method leaves everything practically unstained but the organisms and their capsules. Of course, if blood cells are present they take the stain to some extent.

With the copper sulphate method the serum is stained, but is contracted into threads and masses, and does not give a uniform background. (Figure 2.)

The following experiment made with a bacillus isolated in company with a streptococcus from a case of endocarditis, but probably having no etiological significance, shows the advantage in staining capsules of preparations made with serum or serum mixtures. A smear was made directly on a cover-slip from an agar culture, no diluting fluid being used. By the potassium carbonate method a small capsule was demonstrated. When, however, it was mixed on the cover-glass with some serum (glycogen serum) and stained by the same method, very large capsules were found. In some instances the bacilli could be seen dislodged from their central position in the capsules and sticking half way out of them; in other instances the bacilli were seen entirely free and lying naked alongside of the empty capsules. The photograph of this specimen was made from a preparation stained with aqueous gentian violet, washed with 0.25% sol. of potassic carbonate, dried and mounted in balsam. (Figure 12.)

DEMONSTRATION AND STAINING OF CAPSULES ON STREPTOCOCCI.—In determining the growth of the streptococci in the various experimental media, the cover-glass preparations were often stained by the same method (K_2CO_3) as that used to demonstrate the capsule of the pneumococcus. In this way the fact was brought to light that the streptococci of the cultures tested were possessed of capsules, which stained or were made visible by these methods. This was especially true of cultures in sugar serum media, and in the more alkaline serum media. In some cultures the capsules were quite as sharp and as well-defined as those of the pneumococcus; in others

they were less well marked, and in some appeared as if in a semi-fluid state and on the point of dissolving. In these last, when several organisms were massed together, their capsules seemed to coalesce. This, however, may occur with pneumococcus capsules, though it is by no means so frequent.

Source of the Streptococcus Cultures Used in these Experiments.—The source of the various streptococci which have been used in the experiments will be of interest in this connection, since, as was noted in the earlier part of this paper, the presence of capsules on certain streptococci has been looked upon as a character sufficient to place them in separate species.

The cultures enumerated are those which have been used in all of the cultural and capsule tests.

No. 1. Isolated from milk, May, 1898. Has been cultivated on artificial media for many months.

No. 2. Isolated from human throat (diphtheria suspected) November 18, 1898. Has been long on artificial media.

No. 3. Isolated from an abscess of a horse following subcutaneous injection of diphtheria toxin (filtered), May 17, 1900. Some months at least on artificial media.

No. 4. Isolated from another antitoxin horse. Nasal discharge due to disease of nasal sinus, probably necrosis of bone. November, 1901.¹²

No. 5. Isolated from a case of suppurative pylephlebitis in man. This organism accompanied a bacillus which was a strict anaërobe.¹³

No. 7. From a case of appendicitis.

No. 8. Culture marked "M" from the Research Laboratory, Department of Health, N. Y. Isolated from a case of erysipelas.

No. 9. Same culture as "8," but grown for many months as a separate culture under slightly different cultural conditions.

No. 10. Said to have come from a case of scarlet fever. Research Laboratory, Department of Health, N. Y. Marked "B."

No. 11. Present in pure culture in the urine of a man suffering from a marked cystitis. Patient gave history of chronic pyuria. Capsules on streptococci in the original urine smears.

¹² I am indebted to Dr. Theobald Smith for these four cultures. They were labelled I, IV, VII, X in the order enumerated in the text.

¹³ See Norris, *Journal of Medical Research*, 1901, I, 97. I am also indebted to Dr. Norris for culture 7.

No. 12. Rapid ascending cellulitis of leg. Tissues after 24 hours appeared gangrenous. Patient showed marked signs of sepsis, but eventually recovered.

No. 13. Isolated from the heart blood of a patient showing lesions of endocarditis.

No. 14. Isolated from human uterus. Puerperal sepsis.¹⁴

These cultures from such widely different sources present the usual characters ascribed to streptococcus pyogenes, and so far as I have studied them, are not to be differentiated from one another by the ordinary cultural tests. Morphologically they do not present more than the ordinary slight differences which may be probably attributed to slight variations in physical and chemical environment, rather than to constant inherent differences in the organisms themselves.

All of them coagulate milk after a varying number of days, and grow in gelatin at the room temperature. Nos. 8, 9 and 10 developed tardily in gelatin, did not extend along the puncture to the surface of the medium, but grew along the lower half of the line of inoculation. All of the other cultures grew readily in gelatin, some of them spreading slightly away from the point of inoculation on the surface of the medium.

The broth cultures were not especially noteworthy, except in the different behavior of the same organism when tested in broth containing sugars, and in sugar-free broth. Either flocculent growth or uniform clouding may occur according to the character of the broth in which cultivations are made. Uniformly clouding non-sugar broth, they may present flocculi in one or more of the sugar broths, or vice versa, according to the culture tested. These characters seem to be independent of the acid produced in the medium. I note, for example, organism "8," *glucose broth*: uniform clouding, dense; *plain broth*: flocculi, medium amount of growth. Organism "12," *plain broth*: flocculi small, practically uniform clouding; *glucose broth*: flocculi large, plentiful sticky growth, organisms in very long chains.

All of the streptococci from the above described sources have been found to possess capsules which become apparent under various conditions when stained by the methods mentioned. Streptococcus No. 11 had a well-marked and easily stained capsule in the original urine smear preparations, and also in some of the artificial cultiva-

¹⁴ See Wadsworth, *American Journal of Obstetrics*, 1901, XLIII, No. 4.

tions, especially that on glycerine agar, and on serum agar. The same is true of streptococcus No. 7.

The photographs of most of the preparations were taken after the organisms had been cultivated for many generations on artificial media. As a rule, the best examples were given by cultures on ascitic serum agar. My best results with streptococci were obtained with organisms coming from serum (ascitic) agar, and diluted on the cover-glass in a drop of glycogen serum mixture which had been sterilized at 68° C., and had undergone evaporation to about one-half of its original volume.¹⁵ It happened that the chemical and physical condition of this mixture was exceedingly favorable to capsule preservation. Most of the photographs of streptococci were taken from specimens prepared in this way, dried in the air, fixed in the flame, and stained by the method in which a half-saturated aqueous solution of gentian violet and 0.25% potassium carbonate solution are used. Fine clear specimens may often be obtained in this way, when not a trace of capsule can be demonstrated on the organism in smears direct from fluid or other artificial media.

These experiments, in which it has been possible to demonstrate capsules in all of the cultures of streptococci tested and this often after long cultivation on artificial media, suggest that to place streptococci which have been found by the usual methods possessed of capsules, in a different specific group from the ordinary streptococci pyogenes is not warranted. And especially is this true if this character is the sole or major distinguishing feature.

In the present or immediately preceding history of such cultures, conditions favoring the development of capsules may have existed and thus brought the capsule formation to a maximum. Such conditions may have been found in the animal or human host, or as in the case examined by me, in such a medium as an albuminous purulent urine. Organisms coming from such sources, and cultivated on artificial media might well display this character for some time sub-

¹⁵ No attempt has as yet been made to reproduce this medium.

sequently more highly developed than it is among their fellows which had not had the same previous history.

I have noticed that pneumococci, which it is well known have particularly well-marked capsules in rabbit blood or serum, have this character, in some instances at least, to a less degree in the serum of guinea-pigs which have succumbed to the pneumococcus infection.

It seems more advisable, therefore, in the present state of our knowledge, to look upon capsule formation as general among streptococci, and of no absolute diagnostic or differential significance. It is also fair to conclude that unless there are cultural differences accompanying this marked development of the capsule on organisms suspected of being streptococci, that such organisms should not be placed in a separate specific group.

GENERAL SUMMARY AND CONCLUSIONS.

By morphological examination and with current cultural methods a clear differentiation cannot always be made between pneumococci and streptococci. The chief differential character usually depended upon is the capsule of the pneumococcus. Well-marked capsules, however, may occur on organisms which have with reason been classified as streptococci. On the other hand, capsules may not be demonstrable on pneumococci by the usual methods, especially when growing on artificial culture media.

The usual cultural characters and reactions are at best not diagnostic, and are subject to variations which may render them useless as evidence of specific difference.

The experiments recorded in this paper, however, afford some evidence that there are well-marked differences between the metabolic activities of pneumococci and streptococci, which may prove useful in the differentiation of these organisms. These differences in metabolism become apparent when the pneumococci and streptococci are cultivated in an alkaline serum medium, or in a serum medium to which the carbohydrate, inulin, has been added.

Pneumococci slowly produce acid in the alkaline serum.

In the inulin media they ferment the inulin and thus rapidly give rise to acid. Streptococci do not form appreciable acid in either of these media, nor do they ferment the inulin.

The differences between the metabolism of pneumococci and streptococci are indicated by visible changes in the media. Thus the alkaline serum and the inulin serum are coagulated by the acid formed during the growth of the pneumococci. This coagulation is rapid in the inulin serum medium, slower in the alkaline serum medium. The streptococci, on the other hand, do not bring about a coagulation of these media.

We have, therefore, in either of these two media, the alkaline or the inulin, so far as our experience goes, a definite means of differentiating pneumococci from streptococci.

Starch and glycogen media, prepared in the same manner as the inulin medium, are coagulated by pneumococci and by some at least of the streptococci. With the streptococci the coagulation, if it occurs at all, is usually long delayed. It may be that some or all of the streptococci do not ferment pure starch and glycogen.

Lactose, saccharose and maltose are fermented by pneumococci with the production of acid, thus giving rise to acid coagula in media containing serum. Certain members, though probably not all, of the *Streptococcus pyogenes* group ferment these disaccharids—lactose, saccharose, maltose—hence such sugars are not available in media used to differentiate pneumococci from streptococci as a group.

Monosaccharids—dextrose, galactose, possibly all monosaccharids—are fermented by pneumococcus and the various members (probably all) of the streptococcus group. Serum media containing these sugars are rapidly coagulated by the resulting acid.

In serum media, especially starch-bouillon-serum, sterilized at 68° C., pneumococci usually develop well-marked capsules. In some of the serum media, streptococcus cultures may at times have demonstrable capsules.

All streptococci examined, which by the usual methods would be classified as *streptococcus pyogenes*, have been found to possess cap-

sules. These were demonstrated by special methods and stains devised during this work.

These methods and stains are also especially applicable to the demonstration and staining of pneumococcus capsules.

In the light of the demonstration of capsules on streptococci, the usual morphological basis of differentiation of streptococci from pneumococci appears insecure.

This is also true of the separation of the organisms described as capsulated streptococci into species distinct from streptococcus pyogenes, or from true pneumococci.

This separation does not seem warrantable unless other and especially well-marked cultural differences are demonstrated, which distinguish such capsulated streptococci from pneumococci or from streptococcus pyogenes. Well-marked examples of organisms which would probably be described as capsulated streptococci have been examined during these experiments. They have been found to correspond to streptococcus pyogenes when cultivated in the media described in this paper.

It is a pleasure to acknowledge my indebtedness to Dr. Edward Leaming for the photographs reproduced in the plates.

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DESCRIPTION OF PHOTOGRAPHS.

PLATE XXIV.

Fig. 1. Pneumococcus I from Loeffler's blood serum. Spread in condensation water. Stained by potassium carbonate method. 2000 $\times$.

Fig. 2. Pneumococcus I from heart blood of rabbit. Copper sulphate method. Mounted in balsam. 2000 $\times$.

PLATE XXV.

Fig. 3. Pneumococcus II from serum agar. Spread on cover-glass in serum. Pot. carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 4. Pneumococcus I from sugar free broth. Spread on cover-glass in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 5. Streptococcus I from serum agar. Spread in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 6. Streptococcus II from serum agar. Spread in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 7. Streptococcus IV from serum agar. Spread in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 8. Streptococcus VII from glycerine agar. Spread in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

PLATE XXVI.

Fig. 9. Streptococcus IX from serum agar. Spread in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 10. Streptococcus XI from serum agar. Spread in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 11. Streptococcus XIV from serum agar. Spread in serum. Potassium carbonate method. Mounted in K_2CO_3 sol. 1000 $\times$.

Fig. 12. Capsulated bacillus from agar. Spread in serum. Potassium carbonate method. Mounted in balsam. 1000 $\times$.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

FIG. 8.

FIG. 9.

FIG. 10

FIG. 11.

FIG. 12.