

## VARIATIONS IN SPECIFICITY AND VIRULENCE OF PNEUMOCOCCI DURING GROWTH IN VITRO.

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Variations in the agglutinability and virulence of certain strains of pneumococci have long been noticed.

Neufeld (1) reported in 1902 that a strain which had previously been agglutinated by his immune serum, after it had been grown for a long time in artificial media, failed to be agglutinated. At the same time it had become avirulent.

In more recent years much work has been carried on concerning the specificity of the various types of pneumococci. In this work the strains chiefly studied have been those freshly isolated from the human body and the constancy of the immunological specificity of these strains over prolonged periods of time has seemed truly remarkable. For instance, a strain has been continuously cultivated at the Hospital of The Rockefeller Institute for 14 years and has retained its type specificity unaltered under the conditions prevailing in the laboratory. However, attention has been drawn from time to time to the possibility of modifications in specificity occurring when certain strains are grown under unusual conditions. The observation of Neufeld already mentioned and a similar finding by Cotoni (2) in 1912 suggested such an occurrence. In 1915 Friel (3) found that when pneumococci were grown in immune serum they became less virulent and became agglutinable and phagocytatable in normal rabbit serum. About the same time Stryker (4) showed in this laboratory that when a specific virulent strain was grown in homologous immune serum it lost its specific agglutinability, the virulence was decreased, and the power to produce capsules was inhibited. She found that by passage through a few animals the strain regained its original properties. However, she was not studying strains derived from a single cell. More recently Yoshioka (5) has drawn attention to the fact that when pneumococci are grown under certain unusual conditions, as on unfavorable media, or at 39°C., or when cultures are allowed to undergo drying, variations in serological reactions appear. The changes noted were a decrease in agglutinability in homologous serum and the appearance of a tendency to agglutinate in heterologous serum. At the same time the strains became less virulent. He also found that these modifications did not appear simultaneously in all the bacteria of the cultures, but that when cultures undergoing modifications were plated, the bacteria from certain colonies showed

these alterations in a greater or lesser degree, while the bacteria from other colonies remained constant. He also detected certain characteristic features of the colonies permitting the differentiation of the original and variant organisms.

In 1921 Arkwright (6) observed that, under certain conditions, variations occur in old cultures of bacilli of the intestinal group, and he described two forms of colonies. He designated one type as the "S" colony on account of its smooth surface, and the other type as the "R" colony on account of its rough and irregular surface. Similarly De Kruif (7) has demonstrated two kinds of colonies in cultures of *B. leptosepticus*; one type, "D," growing diffusely in broth and being highly virulent for rabbits, and the other type, "G," growing granularly in broth and having low virulence for rabbits. These types remained constant and retained their characteristics during prolonged cultivation in serum and plain broth. Cowan (8) has also observed colony differences in cultures of streptococci. She found that bacteria from the "S" or smooth form of colony were virulent, and those from the "R" or rough form of colony were avirulent for laboratory animals. Bacteria from the different types of colonies also differed greatly in antigenic properties.

Andrewes (9) has noted two varieties of colonies in cultures of bacteria of the Salmonella group. He found that all of the colonies belonged in one or the other of two sharply defined groups. Although the colonies could not be differentiated morphologically, bacteria grown from these colonies were found to differ markedly in agglutinogenic action. Blake and Trask (10) stated that differences are demonstrable among the organisms of a given strain of pneumococci. These observers have found three distinct forms of colonies. The bacteria from colonies of one of the forms differ from those of the other forms in virulence and agglutinability. Griffith (11) has also studied the colonies of pneumococci and the modifications produced by growing pneumococcus in homologous immune serum. When pneumococci grown under these conditions are plated there appear two forms of colonies which he designated as "R" and "S." He considers the "S" form of colony as that of the original unchanged organism, and the "R" colony as that of a variant arising under the unusual conditions of growth. The "S" colonies have a smooth surface and the bacteria forming them produce specific soluble substance in broth culture, agglutinate with specific serum, are virulent for laboratory animals, and on injection into rabbits stimulate the production of immune substances. The "R" colonies have a rough surface and the bacteria which form this type of colony produce no specific soluble substance, agglutinate atypically, and are avirulent. Bacteria of the "R" colonies may revert in all respects to those of the "S" form, or they may remain stable for many generations. For detecting these differences in colonies he recommended an opaque chocolate agar to which red cells treated with chloroform were added.

In the present study are recorded certain observations on the variations induced in pneumococci by growth in artificial media, and the results of attempts to correlate these differences.

*Methods.*

A strain of Type I pneumococcus was used in all the experiments. The original culture killed white mice in a dose of 0.0000001 cc. of a young broth culture.

The bacteria were grown in plain bouillon, and in bouillon to which varying concentrations of bile, or homologous or heterologous immune serum were added.

Successive transfers were made by inoculating 0.5 cc. of an 18 to 24 hour culture into 5 cc. of the medium.

Repeated transfers of the culture were also made in media containing bile. As is well known, bile is lytic for virulent pneumococci, but Avery<sup>1</sup> has found that by gradual adaptation to growth in media containing bile, pneumococci may live and even multiply in the presence of high concentrations of bile. Repeated transfers were made at 4 hour intervals by inoculating 1 cc. of culture into broth containing bile in a dilution of 1:400. Later, the concentration of bile was increased to 1:200 and 1:100, and transfers were made at longer intervals. Finally, after 69 transfers growth could be maintained in broth containing bile in concentration of 75 per cent.

For detecting differences in colonies, plain agar containing 2 per cent of unheated rabbit blood has been used. The chocolate agar recommended by Griffith has, in our hands, not shown any superiority over plain blood agar.

In studying the characteristics of colonies the plates were examined with a compound microscope, with a No. 6 ocular and a 32 mm. objective. The plates were tilted on the microscope stage so that the light was reflected from the surfaces of the colonies. Differences in colonies were found to be most marked after 12 to 36 hours incubation of the blood agar plate. Colonies showing gross and microscopical peculiarities were selected and transferred to blood agar slants for further study.

The immunological specificity of the strains isolated from individual colonies was tested by the so called thread reaction. This test is made as follows: 0.05 cc. of the broth culture to be tested is added to each of a series of tubes of broth containing varying amounts of specific antipneumococcus sera of the various types. The serum dilutions in the broth ranged from 1:10 to 1:16,000. The cultures were incubated for 12 hours. Under these conditions specific organisms in homologous immune serum grow in threads forming clumps which are precipitated to the bottom of the tube as a solid disc. In heterologous serum they grow diffusely. Organisms which are not type-specific exhibit a granular growth in immune serum of all types. This reaction is more delicate than the reaction of agglutination which is usually employed to determine specificity.

*Method of Single Cell Isolation.*—In certain experiments it was found important to study cultures which had grown from a single isolated bacterial cell. For this purpose the following method was employed. The well of a deeply hollow ground glass slide is nearly filled with dextrose agar. After this becomes hard it is inoculated by spreading over it a dilute suspension of the bacteria made by mix-

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<sup>1</sup> Avery, O. T., personal communication.

ing a loopful of the broth culture to be tested with 0.5 cc. of plain broth. The well of the slide is rimmed with vaseline, a cover-glass is placed on this, and the preparation is allowed to stand for from 15 to 30 minutes to permit sedimentation of the cocci. The slide is then placed on a microscope stage and the cover-glass removed. By using the high dry lens a single diplococcus is selected and centered in the field. No difficulty is experienced in locating the bacteria, which are distinctly visible on the agar. The slide is fixed in place on the stage by running a drop of immersion oil beneath it. After the objective (still in focus) is turned away from the preparation the cover-glass is replaced, and the microscope with the slide fixed to the stage is placed in the incubator at 37°. The microscope is brought out into the light from time to time to observe changes. For this purpose the cover-glass is removed and the objective is swung into place. The presence and growth of any unnoticed diplococci in the selected field can also be detected during this period. After about 12 hours the colony developed from the single diplococcus attains a size large enough to be readily seen with the low power objective. It can then be easily touched with a needle and a transfer made to other media.

This method seems to be less tedious than some of those previously devised for single cell isolation and requires little experience. Several difficulties, however, may arise; namely, (1) the cell selected may not grow; (2) other cells may pass unnoticed in the field selected and the colonies arising therefrom may grow together; (3) the culture may not grow when transferred to other media; (4) only one cell at a time can be isolated unless several microscopes are used. Nevertheless, with care and a moderate amount of skill, growths which are quite certainly from a single cell can be obtained in a large percentage of attempts.

#### EXPERIMENTAL.

*Variations Induced by Growth in Plain Broth.*—The culture was transferred 240 times in plain broth. On agar plates made from the last culture two forms of colonies were seen. The colonies from one (S) were flat, thin, greenish, and translucent. Observed under the microscope they had a smooth, shiny surface. When touched with a platinum loop the colony appeared to be of a gelatinous consistency. The other kind of colony (R) appeared heaped up, thicker, more opaque, and less green than colonies of the other kind. Examined under the microscope the surface of these colonies was dull or finely granular. When pushed with a loop the colony seemed quite coherent and moved along as a whole. Occasionally the S colonies also had a granular surface but it was much less coarsely granular than that of the R colonies. Changes in color of the blood about the

colonies due to methemoglobin formation were seen with both kinds of colonies. No intermediary varieties of colonies were seen.

On the plates made from this culture the relative proportion of S to R colonies was approximately 3 to 1. Cultures were obtained by

TABLE I.

*Clumping Reactions and Virulence of a Culture after 240 Transfers in Broth, and of the R and S Forms Derived from It.*

Dilution of serum.	Whole culture after 240 transfers in broth.				S colony culture.				R colony culture.			
	Antipneumococcus serum.			Normal horse serum.	Antipneumococcus serum.			Normal horse serum.	Antipneumococcus serum.			Normal horse serum.
	I	II	III		I	II	III		I	II	III	
1:500	++	++	++	-	++++	-	-	-	++++	++	++++	-
1:1,000	+	+	++	-	+++	-	-	-	+++	++	++	-
1:2,000	=	+	++	-	++	-	-	-	++	+	++	-
1:4,000	-	-	+	-	+	-	-	-	+	-	+	-

- ++++ = firm disc.
- +++ = disc easily broken up.
- ++ = coarse clumping.
- +
- = fine clumping.
- = no clumping.

*Virulence Test.*

Amount of culture injected.	Whole culture.	S culture.	R culture.
cc.			
2	D. 24 hrs.	D. 13 hrs.	S.
1	" 15 "	" 16 "	"
0.1	" 50 "	" 31 "	"
0.01	" 34 "	" 31 "	"

- D. = died.
- S. = survived.

isolation of single cells from each kind of colony and the bacteria were tested for virulence and agglutinability. Cultures from S colonies will be spoken of as S strains and from R colonies as R strains. The results of tests of these cultures are given in Table I.

It is evident from the results given in the table that the whole culture which now contained both R and S forms had lost much of the specificity of the original culture since growth in clumps now occurred in all three types of antipneumococcus sera. The pure S strain is type-specific, growing in clumps only in Type I serum, but the pure R strain forms clumps when grown in all three types of immune serum. It has been found that the bacteria-free filtrates of the S culture give specific precipitin reactions when added to the homologous Type I serum. In the case of the R culture this specific precipitin reaction with the homologous Type I serum does not occur. It is now well known (12) that this specific reaction is dependent upon the presence of the so called specific soluble substance which is elaborated by the bacteria during growth. It is evident, therefore, that the R cultures have lost the property of producing specific soluble substance, while the ability to produce it is retained by the S strain and it is probable that this lack of specific immunological properties in the R strain is related to its inability to produce this substance.

The S cultures killed mice in doses of 0.000001 cc. but the R cultures failed to kill mice even in doses of 2 cc. The virulence exhibited by whole cultures is apparently due to the presence of the virulent S forms.

The S strains are readily soluble in bile while the R strains are more resistant to the lytic action of this agent. Strains of both kinds coagulate milk and ferment inulin.

The individual cocci of the virulent culture obtained from an S colony are typical in morphology and staining reactions and show the presence of a capsule. Organisms from the avirulent<sup>2</sup> culture derived from an R colony, on the other hand, vary in size and morphology and show bizarre bacillary and irregular forms.

*Variations Induced by Growth in Broth Containing Bile.*—A virulent culture was repeatedly grown in broth containing dilute bile as

<sup>2</sup> It is realized that it may not be absolutely correct to speak of these cultures as avirulent—even the ordinary saprophytes may produce death of animals under certain conditions. However, we have found it convenient, and we think not unjustifiable, to speak of any strain of pneumococcus as avirulent when the injection of 1 cc. of a young broth culture into the peritoneal cavity of a mouse fails to kill.

previously described. After the third transfer in this medium cultures made on blood agar plates showed two distinct forms of colonies. These resembled closely the R and S forms described above, with the exception that colonies of the R form were usually much smaller than the R colonies previously observed, and they had a brownish hue. Colonies of each kind were selected and the strains isolated from each form were found to have properties identical with those of the R and S strains previously described.

Cultures of the S strain killed mice in doses of 0.000001 cc. but 1 cc. of the R culture failed to kill. After seven transfers in bile broth, 1 cc. of the culture was injected into a mouse which died after 36 hours. From a culture of the heart's blood of this animal approximately 1 S to 5 R colonies developed. This culture was found to be as highly virulent as the original culture before bile treatment, but still showed an unspecific granular growth in the three types of serum. After nine transfers in broth containing bile it was found by plating that only organisms producing R colonies were present.

*Variations Induced by Growth in Type I Antipneumococcus Serum.*—A virulent Type I culture was repeatedly grown in plain broth containing 1 per cent Type I antipneumococcus serum. Subcultures on plates were made from the first four transfers but no differences in colonies could be detected. After the fifth transfer, a few colonies of the R form were found, most of these colonies being of the typical S form. Cultures from the S colonies were all type-specific and they were all of approximately equal virulence, killing mice in doses of 0.000001 cc. in 48 hours. On the other hand, cultures derived from the smaller colonies were all avirulent, failing to kill mice in doses of 1 cc., and they showed marked clumping reactions in Types I, II, and III immune sera.

After successive transfers in homologous immune serum broth, the relative number of R colonies progressively increased and after the ninth transfer only colonies of this form were present. At this time the culture was found to be avirulent.

A second experiment was made with serum in a concentration of 10 per cent instead of 1 per cent as in the previous study. Now, even after the second transfer, bacteria producing R colonies were found to be present.

*Variations Induced by Growth in Heterologous Serum.*—Of the various media used in the attempt to produce variation in Type I pneumococcus cultures, heterologous (Type II) antipneumococcus serum seemed to be the least effective. In fact, in this medium changes were brought about only after prolonged cultivation. The R form of colony appeared only in small numbers after 240 transfers.

*Influence of Animal Passage on Cultures Containing Bacteria of Both R and S Forms.*—It seemed of interest to ascertain whether by passing a culture containing both forms through animals the R form might be made to disappear. For this purpose a culture was chosen containing a preponderance of bacteria of the R variety. 1 cc. of this culture was injected intraperitoneally into each of five mice. The mice were killed with chloroform, 2, 4, 6, 8, and 24 hours, respectively, after inoculation, and cultures were made from the heart's blood of each. These cultures were tested for virulence and agglutinability, and were plated, to determine the relative number of R and S forms present. The results of these tests are given in Tables II and III.

When mice were injected intraperitoneally with a culture containing R and S forms, the relative number of R forms in the cultures from the heart's blood rapidly diminished and after 6 hours the cultures showed only the S forms to be present. It is evident that the specificity of the clumping reaction is directly related to the relative number of S and R forms in a culture.

*Separation of S and R Bacteria by Growth in Heterologous Serum.*—Since bacteria of the S forms are clumped only in the homologous serum while the R forms are less specific and are clumped in heterologous serum as well as in the homologous serum, it was found possible to separate the two forms in a mixed culture by growing the bacteria in heterologous serum. A Type I culture containing both R and S forms was inoculated into broth containing heterologous Type II serum. The R forms grew in clumps and were precipitated, while the S forms remained in suspension in the supernatant fluid and plate cultures from the supernatant fluid showed the presence of S colonies only.

*Studies on Virulence and Specificity of R and S Forms.*—The experiments previously recorded indicate that the cultures of pneumococci derived from colonies of the S form exhibit not only serological specific-







cocci. As previously stated, however, when cultures containing pneumococci of the R variety are injected intraperitoneally into a mouse, the bacteria temporarily appear in the heart's blood and may be cultivated from this source during periods of 3 to 4 hours following the inoculation. Therefore, R cultures have been repeatedly transferred through mice, injecting intraperitoneally, and after 3 to 4 hours making cultures from the heart's blood and reinoculating this intraperitoneally into the next mouse of the series. In this way a culture has been passed through 105 mice without the virulence being increased and without any change in the other characteristics being observed.

Stillman (13) has shown that if mice are intoxicated with alcohol they become more susceptible to infection by inhalation of pneumococci. An attempt was made, therefore, to render a culture of the R form more virulent by passing it through a series of mice, each one of the series receiving 1 cc. of a 10 per cent solution of alcohol intraperitoneally half an hour before the pneumococci were inoculated. The inoculations were made intraperitoneally and cultures were made from the heart's blood. This procedure was carried out through a series of ten intoxicated mice but no increase in virulence of the cultures could be detected.

Wadsworth and Kirkbride (14) have shown that the virulence of pneumococcus cultures in artificial media can best be maintained if rapid transfers, every 6 to 8 hours, are made, and they have shown that by this method the virulence of certain cultures may even be increased. As they were not working with cultures derived from a single cell, probably the cultures studied contained both S and R forms. Nevertheless, it has seemed important to determine whether it might be possible to restore virulence to a pure R strain by this method. Although the R strain has been rapidly passed through many transfers of broth, no increase in virulence has been found.

Felton (15) has reported that he was able to restore virulence to a strain of pneumococcus which had become entirely avirulent. He started with a culture from a single cell and, therefore, if our observations are correct, one which consisted entirely of R forms. The increase in virulence was brought about by prolonged growth in milk media, an automatic transferring device being employed. These

observations we have not repeated as the necessary apparatus has not been readily available.

In our hands it has not been possible to render virulent for mice cultures which contained only R forms. Our work indicates that pneumococci of the S variety are readily and frequently changed into the R form. The reverse change, however, has not been observed. The importance of this finding is obvious, but in the light of Felton's observations our conclusions cannot be considered final.

Attention should be drawn to the observations of Webster (16) concerning the bacillus of mouse typhoid, since his observations in many respects bear an analogy to certain of those recorded in this paper. He has found that mouse typhoid bacilli tend to become avirulent, and thus far he has been unable to restore virulence to the avirulent forms. He has also shown that the avirulent organisms have certain characteristic properties as regards growth, colony formation, and immunological specificity.

#### RÉSUMÉ.

The present work was undertaken to study the changes occurring in cultures of pneumococcus when grown under various unusual conditions.

It was found that when pneumococci are grown in broth containing immune serum or bile, or even normal serum, certain changes occur in the characteristics of the culture, chiefly a decrease in virulence and loss of type specificity. Such changes in the culture may appear even when the culture is repeatedly grown in plain broth or on blood agar plates. Further study has shown that these changes do not represent an alteration in all the bacteria of the culture, but are brought about by a variation involving individual bacteria, probably through the loss of certain properties. These bacteria have been found to form colonies having characteristic features. While the colonies produced by unmodified bacteria have a smooth surface, and are more or less sticky in consistency, the colonies formed by the modified bacteria have a rough surface and are dry and friable. The former colonies have been designated as S and the latter, the atypical ones, as R.

When cultures are made from the S colonies it is found that they

are highly virulent, the bacteria have large capsules, produce the so called soluble substance, dissolve readily in bile, and are highly type-specific. Bacteria cultivated from the R colonies, on the other hand, have no virulence for mice, have no capsules, do not produce the so called soluble substance, are somewhat resistant to solution in bile, and have lost to a large degree their type specificity—reacting in all three types of antipneumococcus immune serum.

Cultures made from a single cell of the S colony, which we have called S strains, when grown repeatedly under a variety of unusual conditions do not continue to be composed entirely of bacteria of the S form, but now may contain a variable number of individuals which have the R characteristics.

It seems evident, therefore, that the R pneumococci may be considered as variants of the S or virulent form. On the other hand, single cell cultures from an R colony have remained constant. It has never been possible to isolate S organisms from the pure R strain and it has been impossible to render a pure R strain type-specific and virulent for animals.

Of much significance is the fact that the changes occurring appear to be sudden and abrupt; no bacteria showing intermediate degrees of change have been isolated. Pure cultures of the S strain have remained virulent for mice, pure cultures of the R strain have no virulence (at least under 1 cc. of culture).

That certain cultures of pneumococci may show varying degrees of virulence seems to depend upon the fact that the cultures may contain varying relative numbers of bacteria of the S and R kinds, the degree of virulence depending on the relative proportion of the two kinds. When the culture, as is ordinarily the case, is made up of a great preponderance of S forms the culture is highly specific and on passing the culture through an animal the R bacteria present are rapidly removed. It is only when the cultures are grown under certain conditions, such as these that we have employed, that the R forms become predominant and the characteristics of the whole culture become profoundly modified. Rarely, as we have found in our cultures in bile medium, and homologous immune serum medium, the S organisms may be entirely eliminated and the original characteristics of the culture cannot be restored.

It is not our purpose to discuss here the significance of those findings on the epidemiology of pneumococcus infection. Further studies are required to determine whether variants ever appear in the animal body and if so, under what conditions.

#### SUMMARY.

When pneumococci are grown repeatedly in broth containing immune serum or bile, they become less virulent and less type-specific. These changes are apparently due to the fact that under the conditions mentioned a number of organisms appear which have lost certain properties. These variant organisms form colonies differing in appearance from the colonies of the typical organisms, and cultures made from the atypical colonies are avirulent and are not type-specific. The degree of modification of the original culture is directly related to the relative number of organisms that have undergone variation. After prolonged growth in bile or homologous immune serum variation may have become manifest in all the bacteria of the cultures and the change is then irreversible. The significance of these findings as regards the epidemiology of pneumococcus infections is noted.

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