

A COMPARATIVE STUDY OF SMOOTH AND ROUGH PNEUMOCOCCUS COLONIES.

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PLATES 31 AND 32.

(Received for publication, July 7, 1927.)

INTRODUCTION.

Our knowledge of the production of variant pneumococcus forms has been greatly clarified during the last few years by recognition of the fact that the species may be divided into at least two great groups including the so called S, or smooth forms, and the R, or rough forms. Although the terms rough and smooth have been derived essentially from the morphological characteristics of the colonies formed by respective organisms of each group, they also bear a significant relationship to virulence, serological, and other properties exhibited by the organism. This general concept of bacterial dissociation into rough, smooth, and possibly other groups has not only facilitated the classification of pneumococcus variants but also those of other forms. It has found application in a great variety of bacterial species and, as has recently been emphasized by Hadley's review (1), has become of fundamental importance in bacteriology.

With the pneumococcus a number of features have been recognized as characteristic of the R strains, serving to differentiate them from the S or parent strains. Primarily the surface colonies formed by R strains are atypical, presenting a rough surface in contrast to the typical smooth surfaced colonies of the S forms (2-6). Other recognized properties of the R pneumococcus include those of avirulence for mice,* loss of capsule, inability to produce the so called pneumo-

* The definition of avirulence has in this instance been based upon the fact that 1 cc. of an early broth culture when injected into the peritoneal cavity of the mouse fails to kill.

coccus soluble specific substance and consequent loss of type specificity (7), and an increased resistance to the lytic action of bile (8). Fairly extensive studies have been made on the immunological and other properties of the R pneumococcus, notably those of Reimann (5, 7); the colonies, however, have received more limited attention, and, as our ability to recognize the R forms is in some measure dependent upon the appearance of the colonies which they produce, we have turned our attention to this phase.

It is quite evident at the outset that such a study presents a number of variable factors in that bacterial colonies are structures of somewhat labile nature particularly in the case of an organism such as the pneumococcus, which is extremely susceptible to changes in its environment, resulting in a colony expression which may be quite different in different environments. Our colony studies have been consequently restricted to a comparison between those produced by a small number of standard S and R strains observed under a limited number of cultural conditions. We have not included any of the intermediate strains which have been described by Yoshioka (3) and Blake and Trask (4).

Methods.

The smooth or S strains of type-specific pneumococci were obtained in all instances from the sputum of cases of lobar pneumonia which occurred at the Pennsylvania Hospital during the winter of 1926-27. All of them were subjected to one animal passage and have been subsequently kept at room temperature on rabbit blood agar, with weekly transfers.

The rough or R strains of pneumococci, R-I and R-II, were obtained from The Rockefeller Institute for Medical Research, through the kindness of Dr. O. T. Avery. A third strain which we have designated as "R-III" was isolated from a strain of Type III pneumococci which invariably produced rough colonies when plated upon acid media. These strains were kept in a similar manner on rabbit blood agar.

Strains of *Streptococcus viridans* and hemolytic streptococci, used for comparative colony studies, were obtained from recent blood cultures of cases of subacute bacterial endocarditis and hemolytic streptococcus septicemia respectively. In most of the subsequent experiments fourteen strains of organisms have been used, including nine S pneumococcus strains (three of each type), three R pneumococcus strains (R-I, R-II, and "R-III"), one strain of *Streptococcus viridans*, and one of *Streptococcus hemolyticus*.

The anti-S or type-specific antipneumococcus serum employed for the precipi-

tation and agglutination tests was obtained from the Division of Laboratories and Research, New York State Department of Health. Anti-R pneumococcus serum was prepared by the inoculation of rabbits with strains of *Pneumococcus* R-I and R-II according to the method of Cole and Moore (9).

For colony studies we have selected as a standard the surface growth of 24-48 hour plate cultures upon fresh rabbit blood agar (pH 7.8). The colonies have been studied under the low power objective of the microscope by reflected daylight from the tilted surface of the plate. A mercury vapor lamp was used as the light source in making photographs.

*The Smooth *Pneumococcus* Colony.*

In order to appreciate or emphasize the differences between S and R pneumococcus colonies it is important to review briefly the characteristics of the former. Colonies produced by virulent pneumococci when grown upon the surface of suitable media have long been recognized as presenting characteristic appearances. More than 20 years ago Buerger (10) described and emphasized their distinctive features. He confined his attention to surface colonies in 18-24 hour cultures, grown on serum agar. These he described as circular, disc-like, and flattened, with regular contour. When viewed from above the colony surface appeared glassy, often with a slightly depressed center. When looked at from the side or by transmitted light they appeared as distinct milky rings enclosing a transparent center. This form he designated as the "ring type" of colony. Marked variations in colony size, which reached a maximum of 1.5-2 mm. in diameter, and in form were noted, including the round convex types (characteristic of young cultures) and the large, flat, mucoid forms. Other brief descriptions of the colonies of this organism may be found in most recognized text-books of bacteriology.

For general purposes, however, we have found Buerger's descriptions quite applicable to the majority of discrete colonies of type-specific pneumococci which appear in 24-48 hour cultures on neutral agar enriched with 15 per cent rabbit blood. We have also found the small, round, convex colonies (Fig. 1) to be more frequent in the early (16-24 hour) cultures, and the large, flat, and umbilicated or "ring forms," many of which show extraordinary variations in size, more characteristic of the 24-48 hour culture. The outline of the large forms is generally spherical or elliptical, the surface uniformly

smooth or mucoid (Figs. 2 and 3). One may perhaps make a further differentiation of the colonies formed by individual pneumococcus types for it has been recognized that Type III may produce larger and more mucoid colonies more constantly than the other types. However, these large, mucoid colonies do not seem to be distinctly characteristic for Type III and in the long run predictions as to the type of pneumococcus, based on the morphology of the colony, have proved more or less unreliable in our hands.

Rough Pneumococcus Colonies.

Brief descriptions of rough pneumococcus colonies have been made by Griffith (2), Yoshioka (3), Blake and Trask (4), Reimann (5), and Amoss (6). Reimann has characterized them as follows: "The . . . 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."

Amoss has described the rough pneumococcus colonies as follows: "These were small, flat, and compact, greyish white in color, with an irregular surface. When picked with a platinum needle, the colonies were resistant and could be pushed about on the surface of the agar. With a platinum loop they could be removed *in toto*."

In general we have found the 24 hour colonies produced by the rough strains to be somewhat less variable in appearance than the smooth colonies of a similar age although again, different sizes and shapes are encountered in the 24 hour culture. As a rule the R colonies are smaller, generally circular, and there is greater predominance of elevated convex forms as opposed to the flat, disc-shaped or "ring forms" which are so characteristic of the S colonies (Fig. 4). In fact this tendency for central elevation rather than umbilication recalls the type of colony formed by certain strains of streptococci. In the 24 hour growth the surface is only slightly granular and the difference between the S forms at this stage is frequently not pronounced. After 48 hours, however, the roughening and granularity of the surface becomes more distinct (Fig. 5). At this stage the R

colony appears to be slightly more compact and opaque than the S, but in general the most prominent differences are those of size, shape, and texture of the surface. Subsequently these characteristics become even more pronounced and will be discussed later on in this paper.

The degree of methemoglobin formation about the R colonies may approximate that seen with the S. With some R strains, however, we have noted a tendency for the production of a small halo of hemolysis.

EXPERIMENTAL.

It is evident that typical surface colonies are only produced under optimum cultural conditions and their morphology may, of course, be influenced or altered by a host of incidental or extraneous factors, particularly in the case of an organism such as the pneumococcus which is so sensitive to its environment. Observations on the surface colonies produced by S and R strains of pneumococci under a small number of varied environmental conditions have therefore been included in this study, not only for the purpose of determining some of the conditions which promote or inhibit the formation of typical colonies, but also to familiarize ourselves with the different colony expressions which S and R strains may exhibit under varying conditions. The influence of the following factors has been observed: crowding, age, diminution of the blood in the culture media, and variation in the hydrogen ion concentration of the media. In the latter instances our observations have been limited to a study of the effects produced by relatively sudden changes in environment and we have not studied the colonies produced by organisms which have been grown repeatedly in altered media.

Crowding.—Our attention has been hitherto confined to the study of the discrete colony. It is evident, however, that with most bacteria the morphology of the growth upon an agar surface is greatly influenced by the heaviness of the inoculum and individual colony formation gives way to a confluent growth under conditions of moderate crowding. S pneumococci present no exception to this rule. When small numbers of individual colonies occur in sufficiently close contact so that their edges touch one another, they generally merge to form

confluent dumb-bell- or clover-leaf-shaped forms. With larger numbers and closer contact, a smooth surfaced growth may result in which all trace of individual colonies is lost except at the edges. If, however, the degree of crowding is extreme, large portions of the central areas of the growth may present quite a different appearance. Individual colonies give way to an irregular, amorphous, slightly elevated mass in which there may be myriads of tiny structures with irregular and roughened surfaces. One may note various zones in this process by observing the different aspects displayed in heavily seeded areas from the center out to the edge, where the typical colonies begin to appear (Fig. 6). In the irregular central mass sometimes one encounters irregular colonies with roughened surfaces which are comparable in appearance to those produced by true R pneumococci but under these conditions the presence of such colonies does not indicate that complete dissociation has taken place, for if transfers are made from such areas, typical smooth colonies are produced.

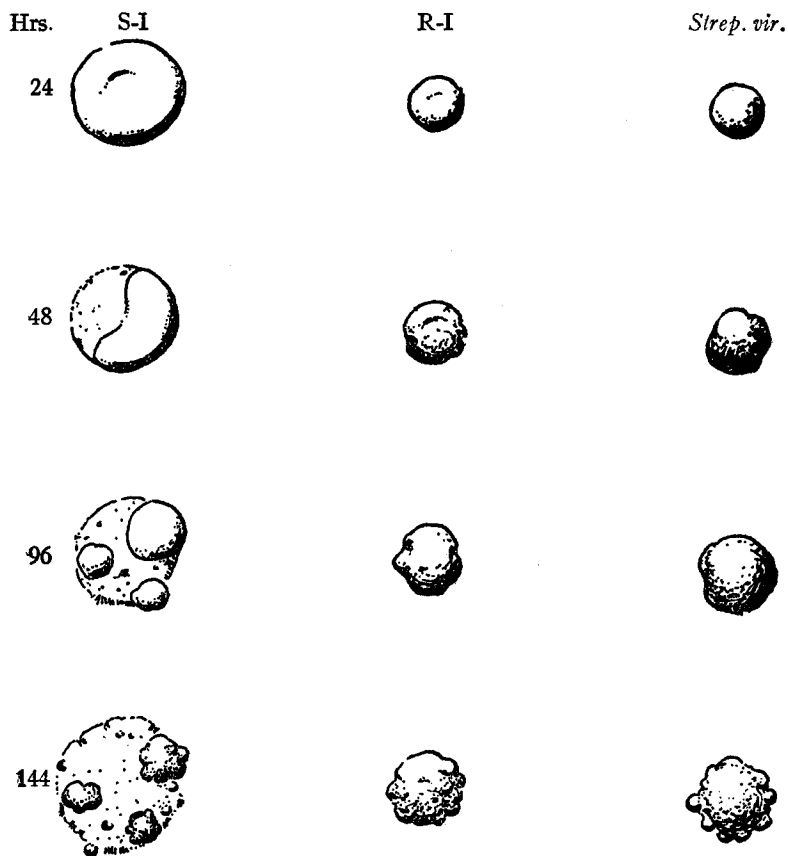
With R pneumococci the growth reaction to crowding is slightly different although the differences are relatively insignificant. The colonies tend to remain discrete to a degree which recalls the behavior of various types of streptococci. In heavily seeded areas individual colonies become reduced in size but still show less tendency to become confluent than does the S variety. With extreme crowding, however, confluence and amorphous growths occur, in which individual colony formation is lost, simulating the changes noted in the crowded S cultures.

Age.—It has long been known that striking morphological changes may be noted in the smooth pneumococcus colony if observed over the course of several days or a week. Such changes are by no means unique for the pneumococcus.

Hadley (1) has reviewed the extensive literature on this subject, emphasizing the phenomena of secondary and even tertiary colony formation which is common to a large number of bacterial species and may occur either with or without actual lysis of the primary colony.

Atkin (11) has recently emphasized some of the changes which the pneumococcus colony exhibits on serum agar plates of suitable reaction and of sufficient depth of media to allow growth to proceed for a considerable time, observing that the colony after increasing in size for 2 or 3 days subsequently became more or less completely autolyzed and transparent. On the succeeding day or two, evidences

of secondary colony formation were observed in which one, two, or more minute papillæ could be discerned on the surface of the original colony and these continued to grow for a week or two, eventually forming opaque secondary colonies which sometimes entirely covered the remains of the original site. He noted that these papillæ or secondary colonies did not undergo autolysis and that organisms from them were resistant to the lytic action of bile.



TEXT-FIG. 1.

In a series of daily observations upon *S. pneumoniae* colonies grown upon rabbit blood agar (pH 7.8) with sufficient depth of the media in the plate to retard drying for a period of a week, we have observed the changes which the colony exhibits during successive days and in particular the processes of colony autolysis and "papilla"

formation. The progressive changes exhibited by both S and R colonies, together with the similarity which the latter bear to the colonies produced by *Streptococcus viridans*, are illustrated in the diagrammatic sketch shown in Text-fig. 1.

As mentioned above, the typical "ring forms" are characteristic of the 24 hour S colony. At the end of 48 hours many of the colonies, having reached their maximum size, begin to show partial autolysis, which becomes progressive during subsequent days. The extent to which this process occurs differs with individual strains and is enhanced when the colonies are in close approximation to one another. At the 48 hour stage, however, frequently the central areas, comprising one-third or one-half of the original colony, appear to have "collapsed" while the remainder still retains its original characteristics, with rounded edges and a smooth surface. The collapsed or autolyzed portions are only slightly elevated from the surface of the media and present a granular or pitted surface, the line of demarcation between the two parts of the colony being generally quite sharp. After the 3rd day the majority of smooth pneumococcus colonies go on to complete autolysis without surviving papillæ and eventually appear under the microscope only as faint shadows (Fig. 7), although to the naked eye they are still visible upon the agar surface. On the other hand, by and after the 3rd day, signs of secondary growth may begin to appear in the form of papillæ, either in the surviving elevated portions or on the remainder of the colony site. These papillæ become more prominent on subsequent days. They are generally multiple and of variable size retaining at first a rounded contour and smooth surface (Fig. 8). With some strains there is a marked tendency to produce large numbers of papillæ so that the original colony site may be studded with dozens of tiny round, conical elevations (Fig. 9). Such papillæ seldom undergo lysis. They may steadily increase in size during the 4th, 5th, and 6th days, extending beyond the edges of the original colony site, thus producing in some instances secondary colonies, as large or even larger than the original. By the 6th day the surviving papillæ or secondary colonies are quite characteristic. If they are large at this stage their surfaces are frequently granular, often rugose, and dotted with protuberances. The edges are beaded with tiny nodules and the picture is not unlike that of an old R pneumo-

coccus or an old streptococcus colony rising from the remains of an autolyzed pneumococcus colony. Here again, although the secondary colony recalls that of an R pneumococcus and, as Atkin has shown, the actual organisms from such a colony do exhibit variant properties, it is evident that complete dissociation into a new strain has not taken place, for if these rough secondary colonies are transferred to fresh media, typical smooth colonies result.

With true R pneumococci the daily changes exhibited by the colonies over a period of a week differ materially from those of the S variety. The 24 hour R colony has already been described. In 48 hour cultures it is larger; it may be either rounded or flat and occasionally shows a slight central depression. Instead of undergoing autolysis as S colonies do, it continues to grow and the characteristics of roughness, opacity, and compactness become emphasized with increasing age, again recalling the changes exhibited by *Streptococcus viridans* more than those of the S pneumococcus. The colony may continue to increase steadily in size, tending to flatten out during the 3rd, 4th, and 5th days often by spreading out from the base either in the form of a scalloped or beaded edge. Tiny nodules or papillæ appear on the surface or edge of the colony about the 4th day, recalling the appearance of those noted in the S colonies.

Diminution of Blood in the Culture Media.—There are undoubtedly a number of conditions under which atypical pneumococcus colonies may be produced upon media which are deficient in suitable nutritive requirements for luxuriant growth. Our studies have been limited to a series of observations on the effect produced by diminishing the amount of rabbit blood added to nutrient agar.

If the concentration of blood is reduced to a point where the pneumococcus begins to grow poorly, distinctly atypical colonies occur. This phenomenon generally occurs for S types when the concentration of rabbit blood added to nutrient agar falls below 5 per cent. The colonies under these conditions appear small and rough. Here again the organisms from the colonies cannot be designated as true R forms for on transfer to suitable media they produce smooth colonies and may be shown to be type-specific by appropriate agglutination tests.

Acid and Alkaline Media.—Finally the effect which variations in the hydrogen ion concentration of the media exert upon colony formation of both S and R pneumococci has been studied.

The experiment was performed in the following manner: Twelve lots of nutrient agar were prepared covering a range in pH of 6.2-9.4, and to each lot 15 per cent rabbit blood was added. Owing to technical difficulties the final pH of the blood agar was not estimated and we have consequently designated the samples of media at which growth could be initiated on the acid and alkaline side as about pH 7.0 and about pH 8.3 respectively. Daily observations and drawings of the colonies were procured covering the changes exhibited over a period of a week by each strain on the different lots of media.

TABLE I.
Differential Properties of S and R Pneumococcus Colonies.

Property	S	R
Size	Marked variability, ranging from 0.1-2 mm. in diameter. Original colonies reach maximum size between 24 and 48 hrs.	Moderate variability, ranging from 0.1-1 mm. in diameter. Colonies may show progressive increase in size over a period of 5-7 days
Shape	Round or elliptical, often exhibiting a central depression. Tend to be confluent	Round, elliptical, and often irregular. Tend to be discrete
Surface	Smooth during first 48 hrs. with subsequent irregularity accompanying autolysis	Becomes progressively rough after 24 hrs.
Autolysis	Marked in 36-96 hr. cultures	Practically absent during 1st wk.
Secondary colony formation	Marked in 36-96 hr. cultures	Present in 48-96 hr. cultures
Methemoglobin formation	Present	Present, but may be replaced by slight hemolysis

From this study we have made the following general observations. On the extreme alkaline side (pH about 8.3) the colonies of S strains of pneumococci proved to be quite atypical, small, and rough, resembling somewhat the colonies produced by these same organisms when grown upon media poor in blood. Here again, however, the organisms could not be classified as true R pneumococci for they showed colony autolysis, and if transferred to more suitable media, immediately produced typical smooth colonies. On the acid side the

S pneumococcus colonies showed little deviation from the normal picture with the possible exception of the fact that with certain strains of Type III the colonies occasionally underwent more rapid lysis coupled with early and extensive formation of secondary colonies, a phenomenon which has been noted by Atkin (11). An interesting feature, however, was that with one of the Type III strains typical, rough colonies appeared on the 2nd or 3rd day, scattered among the smooth whenever it was plated upon acid media. The organisms from these colonies proved to be true R pneumococci.

With R pneumococci, the colonies proved quite constant in appearance when grown in media covering a wide range of hydrogen ion concentrations, and apart from the fact that they tended to be somewhat smaller in strongly acid and alkaline media we have failed to note other changes.

General Characteristics of Smooth and Rough Colonies.—Some of the properties which have been found characteristic for S and R pneumococcus colonies have been assembled, for the sake of brevity, in tabular form (Table I). We have only mentioned those which are of more value in differentiating the two groups.

DISCUSSION.

The general appearances and properties of typical S and R pneumococcus colonies have been briefly reviewed with a discussion of the distinctive colony characteristics presented by each group. Emphasis has been laid upon the more labile nature of the S colony which is characterized by the rapid growth of a smooth surfaced disc-shaped structure which subsequently undergoes rapid autolysis and secondary colony formation. The R colony is characterized by a rough surface, gradual and progressive increase in size over a period of several days, failure to undergo rapid autolysis in early cultures, and more limited secondary colony formation, recalling in some measure the appearance and behavior of the colonies produced by members of the *Streptococcus viridans* group.

It has also been shown that mere roughness of the surface of a pneumococcus colony does not necessarily indicate that the organisms from such a colony are true R forms for some of the observations given above show that S pneumococci may, under a variety of different

environmental conditions, give rise to "pseudo rough" colonies. The distinction between "pseudo rough" and true R colonies is that organisms from the former retain their type specificity and immediately give rise to typical S colonies when transferred to suitable environment, whereas, it is recognized that the organisms from true R colonies do not revert to S forms with such apparent ease. It is conceivable, however, that the organisms in "pseudo rough" colonies may be closely related to some of the intermediate forms which have been described.

SUMMARY.

The characteristic appearances exhibited by the surface colonies of both S and R pneumococci in 24 and 48 hour cultures upon rabbit blood agar have been reviewed. Emphasis has been laid upon the behavior and structure of the colonies formed by R pneumococci, their frequent similarity to the colonies formed by certain strains of *Streptococcus viridans*, and their failure to undergo rapid autolysis in the first 48-96 hours, a phenomenon which is highly characteristic of the S pneumococcus colonies. With the S pneumococci it has been shown that "pseudo rough" colonies may be immediately produced under certain unfavorable cultural conditions but such changes in colony morphology as these do not indicate that complete dissociation has taken place and that the organisms may be classified as true R pneumococci.

In conclusion I wish to express my thanks to Miss Margaret McClintock for her assistance in the technical work of this study.

BIBLIOGRAPHY.

1. Hadley, P., *J. Infect. Dis.*, 1927, xl, 1.
2. Griffith, F., *Rep. Pub. Health and Med. Subj., Ministry of Health, No. 18*, 1923, 1.
3. Yoshioka, M., *Z. Hyg. u. Infektionskrankh.*, 1923, xcvi, 232.
4. Blake, F. G., and Trask, J. D., Jr., *Abstr. Bact.*, 1923, vii, 353.
5. Reimann, H. A., *J. Exp. Med.*, 1925, xli, 587.
6. Amoss, H. L., *J. Exp. Med.*, 1925, xli, 649.
7. Reimann, H. A., *J. Exp. Med.*, 1926, xliii, 107.
8. Reimann, H. A., *J. Exp. Med.*, 1927, xlv, 1.
9. Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, xxvi, 537.

10. Buerger, L., *Centr. Bakt., 1. Abt., Orig.*, 1905, xxxix, 20.
11. Atkin, E. E., *J. Exp. Path.*, 1926, vii, 167.

EXPLANATION OF PLATES.

PLATE 31.

FIG. 1. Colonies formed by *S. pneumoniae* (Type II) in an 18 hour culture on rabbit blood agar. Some of the small convex forms show beginning umbilication. × about 20.

FIG. 2. Colonies formed by *S. pneumoniae* (Type II) in 24 hour culture, showing large, flat, disc and ring forms. × about 40.

FIG. 3. Colonies formed by *S. pneumoniae* (Type III) in 24 hour culture showing rounded and elliptical forms. Typical central umbilication is noted in the middle colony. × about 50.

FIG. 4. Colonies formed by *R. pneumoniae* (R-I) in 24 hour culture. Most of them assume a convex form. The tendency for central elevation rather than umbilication is emphasized by the dark shadows surrounding each colony. The surface is definitely granular. × about 50.

FIG. 5. Colonies formed by *R. pneumoniae* (R-II) in a 48 hour culture. Roughening and granularity of the surface are more pronounced at this stage. × about 50.

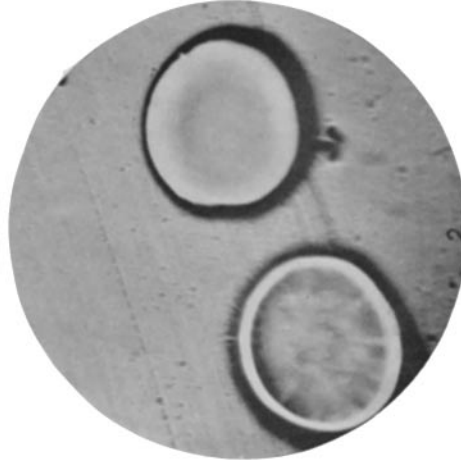
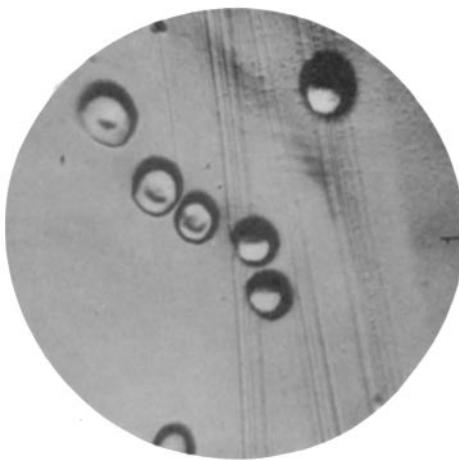
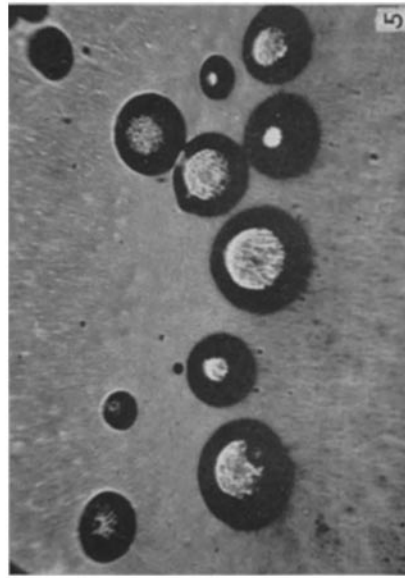
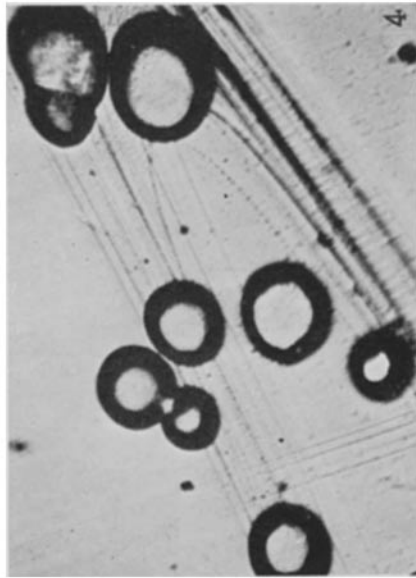
PLATE 32.

FIG. 6. Showing the effect of crowding as exhibited by a 24 hour culture of *S. pneumoniae* (Type II) *pneumococci*. A few typical colonies survive at the edge of the growth. The central areas present an irregular amorphous appearance. × about 20.

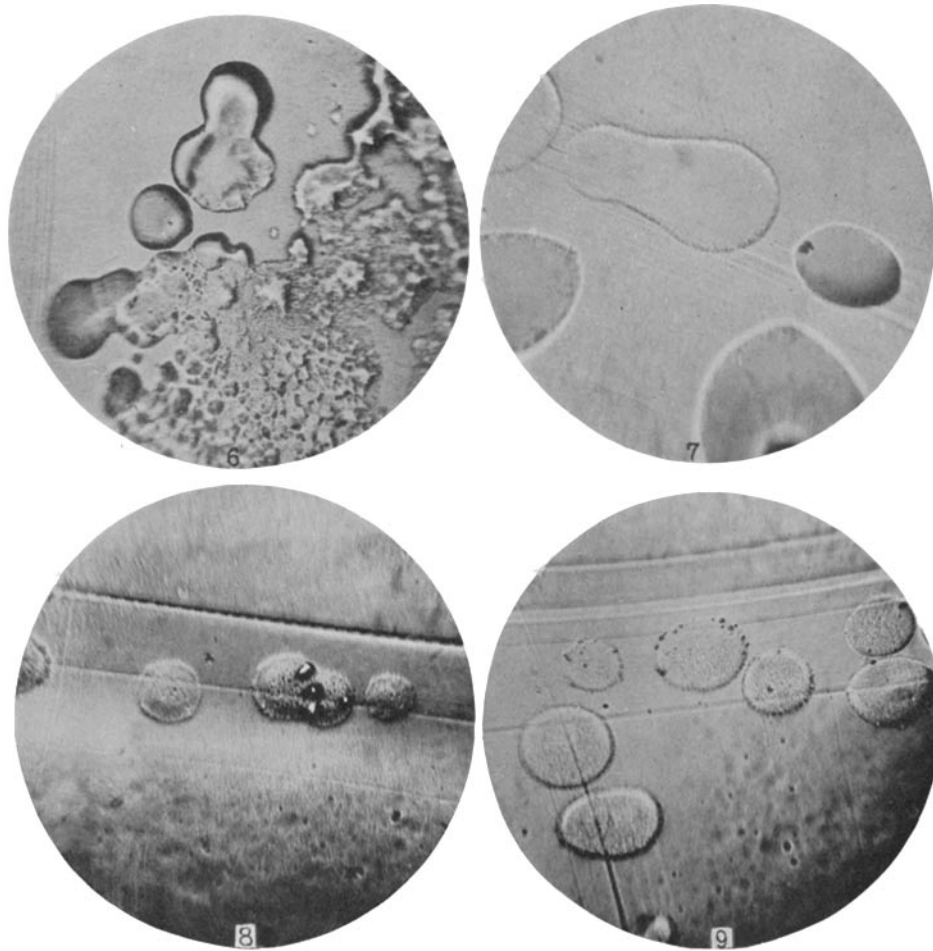
FIG. 7. Colonies of *S. pneumoniae* (Type III) in a 72 hour culture. Moderate lysis has taken place; cf. Fig. 3. × about 40.

FIG. 8. Colonies of *S. pneumoniae* (Type I) in a 72 hour culture showing partial lysis with surviving papillæ on one of the colony sites. × about 30.

FIG. 9. Colonies of *S. pneumoniae* (Type III) in a 72 hour culture showing partial lysis and multiple papillæ about the edges of individual colony sites. × about 30.



(Paul: Smooth and rough pneumococcus colonies.)



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