CHAPTER V

PNEUMOCOCCAL DISSOCIATION AND TRANSFORMATION

Changes in the morphological, cultural, pathogenic, and immunological characters of pneumococci caused by various physical, chemical, and serological conditions in their environment; the transformation of the diplococci from one serological type to another; and the relation of the species to streptococci.

THE constancy of the biological characters of Pneumococcus depends on the conditions of its surroundings. When the conditions are favorable, the morphological and serological integrity of the cell remains stable. Subjected to unfavorable influences, Pneumococcus exhibits great lability of form and function. The form of the cell may pass through every stage from the typical encapsulated diplococcus to one completely denuded of capsule; pathogenicity may be diminished from full virulence to entire absence of infectivity; and antigenic and serological properties may lose strict type-specificity and retain only the broader species-specificity. With the restoration of favorable conditions, the degradation process, if it has not proceeded too far, ceases and is reversed, the cell again assuming its typical characters. But, what is still more remarkable, a degraded coccus originally derived from a fixed type may, under appropriate stimulation, develop the vital and immunological properties of a different specific type. Thus, in addition to natural occurrence of variation or dissociation, the actual transformation of pneumococcal types has been experimentally induced and, possibly, species mutation has occurred. Lack of knowledge of the existence of bacterial variants and of the factors inducing dissociation has undoubtedly caused much confusion in bacteriological diagnosis and in the interpretation of the serum reactions of Pneumococcus.

Early Observations of Dissociation: 1891–1921

Bacterial variation is not a new phenomenon. It is only the study and the explanation of the underlying causes that are recent. In 1891, Kruse and Pansini[763] first called attention to changes in morphology, cultural characters, and virulence of pneumococci under artificial cultivation. Pure cultures freshly isolated from pneumonic material were typical in appearance during early generations but, on continued cultivation on unfavorable media, the cells exhibited deviations from the normal characters. The lance-shaped, diplococcal forms became streptococcal or even bacillary, the capsule rapidly vanished, and virulence waned. The degenerated cocci, when passed through susceptible animals, regained their capsules and virulence, and when returned to favorable media, again showed normal pneumococcal morphology. Kruse and Pansini, therefore, noted many of the features of dissociation and were aware of the first causes of the phenomena.

In the next year, Arkharow[17] reported changes taking place in cultures cultivated in the serum of vaccinated animals. Growth was slow in developing and at the end of three or four days the cocci began to diminish in size, to grow in long chains, and to lose virulence. Four years later, Eyre and Washbourn[373] described the variations observed on continued cultivation of pneumococcal strains in broth. Of one strain it was said that it differed in "morphology, biology and pathogenic properties from the parent stock. It, in fact, represented a distinct variety, possessing practically no virulence, and growing luxuriantly, even at 20°C., on all the usual media." The first attempts to induce this variant to revert to its former state were unsuccessful. Then, by passage through a rabbit, the variant reverted to its original form.

Hiss, Borden, and Knapp (1905)[651] encountered organisms, indistinguishable in fermentative reactions from pneumococci, which showed variations in morphology or agglutination, and the authors considered that the cultures were temporarily or perma-

nently modified pneumococci, *Streptococcus mucosus*, or streptococci of hitherto unrecognized types.

A suggestive communication, because of its anticipation of later discoveries, was that of Rosenow[1163] published in 1910. He presented the results of a study of seven cultures isolated from endocarditis which he believed were "modified pneumococci." All the strains fermented inulin and produced a variable amount of greenish discoloration but no hemolysis on blood-agar plates, but grew atypically with the development of involution forms on media containing the patient's blood. However, cultivation in normal serum or blood and animal passage promptly restored normal pneumococcal characters to the modified strains. Recultivation in the patients' serum brought out the modified characters. These special characters varied greatly in the strains studied; the more chronic the disease process in the patient from whom the serum was obtained, the more marked were the changes. This last observation would seem to argue for the occurrence of variation or dissociation *in vivo*—a biological process concerning which there is still some doubt.

The phenomenon of bacterial dissociation, or as it was known in the early years of this century, variation, received little attention until 1915 when Friel[494] reported that prolonged cultivation of bacteria rendered them more susceptible to phagocytosis. He called the process "Piantication" (fattening for slaughter) and observed its operation with strains of Friedländer's bacillus, *Pasteurella*, and Pneumococcus. The same effect was produced by exposing the organisms to immune serum; while the reverse process took place when "pianticated" bacteria were grown in normal serum—they regained their resistance to phagocytosis.*

In the next year, Stryker[1348] described the variations induced in

* Neufeld and Schnitzer credited the first demonstration of bacterial dissociation to Friel, although Hadley[584] ascribed the discovery to Baerthlein (1912). The phenomenon had, however, been observed much earlier by Kruse and Pansini (1891),[763] and by Arkharow (1892),[17] and had been described in some detail by Eyre and Washbourn[373] in 1897.

Pneumococcus by cultivation in immune serum. When virulent strains were grown in broth containing homologous immune serum, there developed variations in agglutinability, decrease in virulence, inhibition of capsule formation, increase in phagocytability with normal serum, and a change in absorptive power and in antigenic properties. Reversion of the changed forms to the original type took place on animal passage, the number of such passages required usually varying with the number of previous serum treatments. The immune response, as measured by agglutinins, was slower in rabbits injected with strains grown in immune serum than with those cultivated in normal serum. A spread of agglutinative action was evident in the ability of the serum of immune rabbits injected with a serum-treated Type II culture to agglutinate pneumococci belonging to both Types I and II. Type-specificity was being lost and replaced by species-specificity. Cultures grown in normal serum formed capsules upon injection into mice, whereas those grown in homologous immune serum under similar conditions showed no demonstrable capsules. This loss of the ability of Pneumococcus to synthesize the capsular substance was later to assume a new and broader significance.

Later Observations of Dissociation

SMOOTH AND ROUGH FORMS OF PNEUMOCOCCUS

Arkwright (1921),[18] in studying the colony appearance of dysentery bacilli grown on media containing immune serum, gave the designations S and R—smooth and rough—to the dissociants because of the corresponding differences in colony topography of each form. Griffith,[560] in 1923, extended Stryker's observations and applied the letters S and R to the two forms of colonies he observed when pneumococci were grown in media containing homologous immune serum. The S colonies have a smooth surface, and the cocci forming them produce the soluble specific substance in broth culture, agglutinate with specific serum of the homologous

type, are virulent for laboratory animals, and on injection into rabbits stimulate the production of type-specific immune substances. The R colonies have a rough surface, and the organisms comprising them form no soluble specific substance in broth culture, agglutinate atypically, and are avirulent. Cocci of the R colonies may revert to the S form, or they may remain stable for generations. Another property of the S cells is the ability to absorb from immune serum all antibodies for both S and R forms. The R forms absorb only the anti-R bodies, and when injected into animals fail to stimulate the formation of type-specific (S) antibodies.

Griffith considered that the R form was differentiated from the S by the loss of virulence and by the ability to form capsules and to elaborate soluble specific substance, and that the R form represented a stage in the degeneration of Pneumococcus from the virulent, complex type of S cell to an attenuated form with a simpler antigenic structure. Griffith also found that degradation might not be permanent and that reversion could take place after animal passage or repeated cultivation in blood broth. The author recommended for the demonstration of variant colonies an opaque "chocolate" agar to which red blood cells treated with chloroform had been added.

Griffith looked upon the S form as the original, unchanged organism, the R form as a variant due to unusual growth conditions. The degenerative action of immune serum Griffith believed to be a double one. He suggested as an explanation of the change the view that serum might disorganize the biological functions of Pneumococcus by precipitating the capsule, thus inhibiting the secretion of antileucocytic substances and rendering the organism temporarily harmless, and that when pneumococci divided in the animal body in the presence of immune serum, the influence of the serum might cause progressive attenuation of subsequent generations. In connection with the causes for such bacterial variations, Eastwood[344] contributed an interesting theoretical discussion. It would take us too far afield to quote from it here, but the communication is recommended to those readers who desire to learn more of the philosophical aspects of the phenomenon of dissociation.

In seeking a medium that would emphasize the differences in variants, Sia and Chung,[1270-1] by the substitution of normal dog blood for rabbit or horse blood in agar for plate cultures, obtained such marked differences in the morphology of S and R colonies that differentiation, they claimed, became extremely simple. With a moderate degree of magnification (× 28), the S colonies were seen to be smooth and glossy, while the R colonies, including those derived from Pneumococcus Type IIS, revealed a wrinkled and coarsely rough surface. The R colonies also exhibited strong hemolytic properties. Sia and Chung tested the blood of guinea pig, white rat, chicken, and cat, but none was so good as dog blood. These authors believed that the property of dogs' blood resided in the cellular elements rather than in the plasma, and probably was due to hemoglobin.

MODIFICATIONS A, B, AND C

Blake and Trask (1923),[129] in conducting experiments similar to those of Stryker, also found that growth of pneumococci in homologous serum resulted in a marked loss of virulence, accompanied by constant and distinct changes in agglutinative properties with respect both to the character of agglutination and the zone of optimal reaction. The authors found the changes not to be a gradual alteration of all members of a culture but, instead, there appeared to be a comparatively rapid and complete change in individual organisms. The variants exhibited changes not only in virulence and agglutinability, but also in colony appearance, by means of which three modifications, called by the authors A, B, and C, could be distinguished.

In the same year, analogous observations were published by Yoshioka.[1564] Typical pneumococci underwent apparently regular serological modifications when maintained under unfavorable con-

ditions, such as surface cultivation on unsuitable media, incubation at 39°, and too long-continued drying. The same conditions also led to loss of virulence. The changes noted consisted in a marked decrease in agglutinability with homologous serum and in the appearance of an enhanced agglutinability with heterologous serums. The modified strains were, at times, agglutinable by antistreptococcic serum. The changes appeared irregularly and suddenly and did not parallel the degree of decrease in virulence. An immune serum obtained after immunization with an atypical strain agglutinated only that specific variant and not the parent strain.

In the discussion which followed the presentation of papers on bacterial variability before the German Association of Microbiology at Göttingen in 1924, Neufeld[979] reported a change in bile-solubility of pneumococcal variants, as well as in their susceptibility to optochin. In the same year, Felton and Dougherty[425] observed that pneumococci when grown in plain broth in an automatic transferring device suffered a loss of virulence which was directly proportional to the change in the hydrogen ion concentration of the medium—the more acid the medium the greater the loss of virulence. Accompanying the change in virulence there was an alteration in the behavior of the organisms toward agglutinating serums. Although specific, the agglutinability of the modified strains became greater than that of the parent organism.

COMPOSITE CULTURES

Amoss[12] in 1925 published an article on the composite nature of a pure culture of virulent pneumococci from which he derived several strains by the Barber single-cell technique. These were cultivated in broth containing Type I antiserum, and the pure culture was submitted to successive transfers in bile broth and acid broth. Amoss reported that the virulent strain of Type I Pneumococcus, after being passed through 190 mice, was composed of individuals possessing characters differing from those of the original culture. A pure-line strain derived from a single cell isolated from a virulent composite culture was more virulent for rabbits and less resistant to unfavorable media than was the composite strain or other strains similarly obtained from the same source. Amoss isolated by the plating method an avirulent strain from the composite virulent culture which had been repeatedly transferred and grown in immune serum broth, bile broth, and slightly acid broth. Cultures of the virulent single-cell derivative, when grown in these media, also gave rise to the avirulent form. Heterologous immune serum and also normal serum did not favor the change from virulent to avirulent variants. The avirulent strains, however procured, were all of a single sort. They formed characteristic colonies, showed no tendency to revert to the parent type, and did not become virulent on repeated passage through mice. Serum from rabbits immunized with the avirulent variants possessed agglutinins but no protective antibodies for the parent strain. It seems clear from Amoss' experiments that he had succeeded in effecting a permanent degradation of a virulent Type I Pneumococcus, with a loss of type-specificity, but not of species-specificity.

The results of Reimann's[1125] study, published in the same month in which Amoss' publication appeared, agreed with both Amoss' and Griffith's observations. Reimann reported that cultures of pneumococci from single-cell seedings, when grown in broth containing immune serum, bile, or even normal serum, suffered a decrease in virulence and loss of type-specificity. The changes might take place when the pneumococci were repeatedly grown in plain broth or on blood agar, but were due to variations in individual cells, rather than in the cocci of the culture as a whole. Reimann preferred 2 per cent unheated blood agar to Griffith's chocolate agar, and on this medium there appeared characteristic smooth colonies along with others of the rough form. Cultures from S colonies were highly virulent, had large capsules, produced soluble specific substance, dissolved in bile, and were strictly type-specific. Strains from R colonies were avirulent for mice, had no capsule, produced no soluble specific substance, did not dissolve so readily

in bile, and had largely lost their type-specificity. Single-cell cultures propagated from S colonies, after repeated transplants under unsuitable conditions, produced some R variants, while single-cell cultures from R colonies, under the same circumstances, remained constant in character.

SPECIES-SPECIFICITY OF ROUGH FORMS

A few months later, Reimann[1126] published these further conclusions:

> Immune sera prepared with the degraded or variant forms of pneumococci (R strains) are similar in their reaction to sera prepared with the protein or cell solutions of pneumococci. They contain antibodies reactive with the protein of all types of pneumococci, but no antibodies reactive with the type-specific substances. Pneumococci of the variant or R form, regardless of type derivation, are serologically identical and have the antigenic characteristics of pneumococcus protein. They evoke the species-specific and not the type-specific antibodies. Antipneumococcic sera produced by immunization with S strains may contain species-specific antibodies in addition to those which are type-specific. Each kind of antibody can be removed separately from these sera by selective absorption with the R and S strains of pneumococci.

These fundamental observations were later to be confirmed and explained by the discoveries of Avery and Heidelberger of the antigenic chemical constituents of the pneumococcal cell.

Takami,[1378] contemporaneously with Amoss and Reimann, added a few new facts about variation. His study included certain strains that were apparently stable in their original characters, since they gave rise to no variants even after two or three years' cultivation. There were other strains that showed a strong tendency to vary, and in a short time became so changed that they no longer produced any typical colonies. In agglutinative abilities the same rule held true. There appeared to be no direct relation between decrease of agglutinability and atypical colony formation. The only two characters that were closely connected were bile-solubility and

inulin fermentation. When either of these properties was lost the other disappeared.

Takami[1375] followed the *in vitro* experiments with a study of the variations displayed by pneumococci propagated in the animal body. Rabbits, guinea pigs, mice, white rats, and house rats were used for this purpose, and the variants produced in these animals differed in agglutinative characters from the forms developed on artificial media. The explanation offered was that in the body the organisms lose their old receptors and acquire new ones. Takami separated five typical strains of "culture-bacteria" (pneumococci long grown on blood agar) into colonies that were still markedly agglutinable, and into others that had lost this power. The latter were found to be highly virulent for mice, whereas the former were avirulent.

A few years later, Kimura, Sukneff, and Meyer[711] repeated the dissociation experiments, using broth containing 10 per cent homologous immune serum, with subsequent cultivation of the variants on Griffith's chloroform-blood agar and Bieling's blood-water agar, both of which have a laked-blood base. The results were similar to those reported earlier, but the authors believed that they had demonstrated the production of other variants in addition to the atypical R forms with divergent cultural and serological characters. The experimental data, however, are insufficient for judging the claim.

For determining the true character of normal strains and of dissociants, Schiemann[1225] adopted as a criterion the possession of a type-specific (dominant) hapten as a prerequisite for the formation of type-specific agglutinins and protective antibodies and also for virulence. For the recognition of type-specificity the essential considerations were, first, coarse agglutination in homologous antiserum determined by the carbohydrate nature of the hapten; second, the repression of cross-agglutination in heterologous serum; and, third, mouse virulence. According to these standards, in

addition to normal and degraded R forms, Schiemann postulated intermediate variants which he claimed represented pseudo-types. The discussion was largely theoretical, and since he gave no experimental data, it is impossible to judge the validity of his claims.

ELECTROPHORETIC POTENTIAL OF VARIANTS

Falk, Jacobson, and Gussin,[383] and then Falk and Jacobson,[380] studied another criterion for variability. The authors measured the electrophoretic potential of Blake's variants A, B, and C from Type I Pneumococcus during cultivation on blood-agar slants, with weekly to bi-weekly transplants, over a period of one and one-half years. The velocities remained constant and paralleled the virulence of the strains. Although the authors believed that electrophoretic potential was related in some fundamental manner to virulence, phagocytability, agglutinability, and other serological characters of microorganisms, the particular variants studied were indistinguishable from the parent strain in these characters, and after a large number of generations on blood agar showed no evidence of spontaneous changes. The only exception to this stability of character was a single-cell strain of variant C which reverted to the A form on passage through a mouse.

EFFECT OF CHARCOAL, YEAST, OPTOCHIN

The variation in pneumococci appearing after growth in broth containing animal charcoal or dry yeast and subsequently in optochin broth, first observed by Berger and Englemann[100-1] and shortly afterward by Morgenroth, Schnitzer, and Berger,[929] was corroborated in 1927 by Amzel.[14] Cultivation in these media gave rise to rough colonies, the members of which were avirulent for mice and exhibited diminished solubility in bile. One strain developed hemolytic properties, and another became agglutinable with antiserum for the fixed types. The variations observed after cultivating the cocci in the presence of bile were the same as those occurring in the Schnitzer-Berger medium.

In Amzel's[15] next paper it was reported that pneumococci isolated from pneumonia patients before optochin injections were of the smooth type, while the organisms cultivated after injection grew as rough colonies. Untreated cases yielded only smooth colonies and, in two cases repeatedly treated with optochin, the isolated culture was persistently composed of both smooth and rough forms. Amzel attempted to convert the rough into smooth strains by mouse passage but was able to effect this reversion in only one of three trials.

During the 1920's there came abundant confirmation and expansion of the earlier observations on pneumococcal dissociation. Jacobson and Falk (1926–1927),[674-5] continuing their earlier studies, were able to degrade smooth strains of Blake and Trask's A, B, and C modifications into rough strains by growing the organisms in broth containing specific immune serum, although after twenty-three transfers the conversion was incomplete. The cultures were still mixtures of S and R varieties. The former continued to have the same virulence and electrophoretic potential, but the latter were reduced in both virulence and potential. Rough variants of the B and C strains reverted after twelve transfers in homologous immune serum broth, and showed the same virulence and potential as the original smooth organisms. In all the strains studied there were alterations in virulence accompanied by parallel alterations in electrophoretic potential and by reciprocal changes in agglutinability. Levinthal[800] also observed changes in virulence and in the cultural and serological behavior of a highly virulent Type I pneumococcus after cultivation in serum broth. He was able to effect the transformation of R to S forms by growth in broth at 25° and by subsequent mouse passage.

IN VIVO VARIATION

Similar variations apparently taking place *in vivo* were described by Wadsworth and Sickles.[1474] Cultures isolated directly from the blood stream of horses undergoing immunization, or at

the necropsy of animals that had died as a result of the development of pneumococcal lesions in the heart or other organs, exhibited attenuation of virulence, loss of capsule formation, antigenic power and type-specificity, and changed susceptibility to phagocytosis. In the case of some of these variants the specific characters of the original type from which they were derived were quickly restored by one or two mouse passages. In other instances, the organism remained atypical.

Sickles,[1279] in a later study (1932) of pneumococcal strains that had become atypical in the tissues of horses undergoing immunization, in comparison with the typical cultures from which they were derived and with various other typical and atypical strains, found that all the organisms were bile-soluble. The maximal limits of growth, along with the other characters, such as limiting hydrogen ion concentration and peroxide and hemoglobin formation, were similar for the same type culture whether original, degenerated, or reverted. Sickles found only one strain which departed from the general rule and that organism grew at 42°, and survived even after incubation at 43.5°. No other pneumococcal strains studied were alive after twenty to twenty-four hours at 42°.

That Pneumococcus may, however, retain its specific type characters when growing in the animal body was proved by Megrail and Ecker[888] in 1924, who injected mice and rats with suspensions of gum tragacanth followed by a saline suspension of pneumococci. In these fixation abscesses the strains displayed no variation and no change in agglutinability. Here the conditions differed from those in the horses harboring pneumococci, as reported by Sickles,[1279] since the rats and mice had not been subjected to any immunizing treatment, and their tissues, therefore, presumably contained no antibodies which might favor variation.

Reimann[1128] found that R forms occurred *in vivo* but could discover in his experiments no positive evidence that recovery from pneumococcal infection depended upon the degradation of virulent

S forms to avirulent R forms with their subsequent destruction by phagocytosis. In a still later study[1129] it was noticed that daughter colonies frequently appeared among the R variants, and in some instances tended to replace the typical R forms. The daughter strains grew in colonies with glistening surface, morphologically indistinguishable from genuine S colonies, although the characters of the bacteria comprising the daughter colonies conformed to the R variety. Strains of R pneumococci, which had seemed irreversible, were apparently converted into the S form when treated by the method of Griffith, that is, by growth in specific immune serum. Reimann considered that recent experimental studies indicated that virulent S pneumococci might dissociate into the R form *in vivo*, that R forms occasionally could be found in the sputum of pneumonia patients, and also might live dormant *in vivo* for a considerable period of time.

The recovery of R variants from the body has recently been reported by Shibley and Rogers.[1262] Twenty-four lung punctures made in lobar pneumonia patients at the time of crisis or lysis yielded R forms of pneumococci in all but four cases.

DETAILS OF COLONY FORMATION

Dawson[289] maintained that colony morphology alone could not be considered as a final criterion of dissociation; it should be confirmed by specific agglutination and virulence tests. While it was possible, by mouse passage, to accomplish a complete reversion of Type IIR to Type IIS, it was not possible to convert the particular Type IR strain studied to the corresponding S form. In the case of a Type IIIR strain, it required twenty-eight mouse passages to restore the variant to its original S condition. Growth of the same R strains in broth containing 10 per cent anti-R serum resulted in reversion of Type IIR on the fifth transfer, of Type IIIR after eight to twelve transfers, but failed to affect the Type IR. Dawson thought that the reversion of R to S did not depend

on the presence of an admixture of both forms within the culture, but rather that each individual R strain might or might not possess the ability to revert. This varying tendency of R strains was exemplified in one experiment in which one of four other strains of Type IR, obtained by growing a freshly isolated Type IS strain in 25 per cent Type I anti-R serum, reverted to Type IS after forty transfers.

The finer details of colony appearance of R and S forms interested Paul (1927),[1067] who chose a small number of standard R and S strains and studied their growth on agar under a limited number of cultural conditions. Paul described the R colonies as having a rough surface, with a gradual and progressive increase in size over a period of several days and a tendency to remain discrete. The colonies failed to undergo rapid autolysis in early generations and exhibited limited secondary colony formation. Methemoglobin formation was present but might be replaced by slight hemolysis. Paul's S strains grew in rapidly developing disc-shaped colonies with a smooth surface which later showed irregularities. The colonies tended to become confluent and exhibited marked autolysis in thirty-six to ninety-five-hour cultures. In the same period, secondary colony formation took place. Methemoglobin formation was a constant feature.

In a second paper, Paul[1068] gave further information concerning the conditions which affected colony formation. Under extreme crowding, the individual S colonies gave way to irregular, amorphous, slightly elevated masses with myriads of tiny structures having irregular and roughened surfaces, comparable to R colonies, but on transfer to less crowded conditions they developed as typical S colonies. The true R colonies tended to remain discrete, but in dense growth resembled the S colonies under similar conditions. The effect of age on the S colonies was to increase autolysis and papilla formation. With the R colonies there was no autolysis, but roughness, opacity, and compactness became emphasized, with

papillae appearing on about the fourth day. When the blood content of nutrient agar fell below 5 per cent, the S colonies appeared small and rough, yet were not true R colonies. The same effect was brought about by an alkaline reaction of the medium, but the original characters were restored on transplantation to a more favorable medium.

In a study of the bile-solubility of Pneumococcus, Atkin[29] reported that pneumococci growing in papillae or secondary colonies developing on an autolyzed colony from a point inoculation on a thick serum-agar medium of proper reaction were insoluble and that susceptibility of the variants to the action of bile corresponded to the possession by the organisms of autolysin. When the insoluble cocci were subcultured on a fresh serum-agar slant, they regained autolytic properties and bile-solubility.

Grumbach[563] also studied the details of colony formation accompanying the varying degrees of pneumococcal dissociation. He differed with Atkin,[29] but agreed with Paul that daughter colonies were not identical with R forms of pneumococci, because they were never truly granular on ascitic agar, they remained bile-sensitive, were fully virulent, and on transplantation developed into "bud-carrying" S colonies. Grumbach found that the ability to produce hemolysis on blood agar and in blood bouillon in forty-eight to seventy-two hours quite commonly ran parallel with the dissociation phenomena. He described the characters of three virulent S strains isolated from pneumonic material that were not agglutinated by Type I, II, or III serums. Growing for twenty-four hours on ascitic agar the organisms produced the classical picture of pneumococcal colonies. The thickness of the peripheral ring varied, and in one case there was a "wall" formation of the type Buerger and Ryttenberg[169] claimed to have found solely in colonies of streptococci. Grumbach also described a "wing-form" colony which he believed to be similar to that supposed to be caused by phage action on streptococci, and concluded that the same colony

pictures could be obtained for pneumococci as for streptococci, but was not sure how far the bactericidal action of the body fluids or how far bacteriophagic action were to be considered as the basis for the phenomena.

Faragó (1932)[390] investigated the possible participation of bacteriophagic action in the dissociative processes, but decided that it was not a factor. He objected to the designation R and S for dissociants, because secondary colonies were formed from virulent organisms, whereas Griffith's R modification arose from avirulent strains. It is difficult to follow Farago's reasoning, but it may be possible that he had in mind some of the features later described by Dawson.

ANTIGENICITY OF ROUGH FORMS

Tillett[1406] turned his attention to the antigenic properties of the dissociated R forms. When he vaccinated rabbits by repeated intravenous injections of suspensions of heat-killed R pneumococci, the animals acquired a marked degree of active immunity to infection with virulent S forms of Pneumococcus I and II. (Tillett[1404-6] had previously shown that a similar immunization treatment induced active resistance to Type III infection.) Furthermore, the whole citrated blood of the immune rabbits passively protected normal rabbits against infection with Type I and Type III pneumococci, but failed to confer a like protection on mice. According to Tillett this form of acquired resistance to pneumococcal infection elicited by R organisms devoid of type-specificity, and exemplified in animals whose serum possessed no demonstrable type-specific antibodies, presented features which strongly suggested that the underlying mechanism differed from that concerned in type-specific immunity.*

RESPIRATORY CAPACITY OF VARIANTS

Another difference in the character of S and R forms was the

* For a full discussion and bibliography of microbic dissociation up to that time the reader is referred to Hadley's[584] comprehensive article.

changed respiratory capacities of pneumococcal variants. According to Finkle's[440] measurements, the capacities of organisms of Types I and II were altered during conversion from the S to the R form. For Type I Pneumococcus it was increased 110 per cent, while for Type III it was diminished by 45 per cent. In the case of Type II there occurred a diminution of only 16 per cent in respiratory activity. At the same time, anaerobic glycolysis was increased on the average 25 per cent each for all R forms irrespective of type derivation, while Type I Pneumococcus, on being converted to the R form, lost its capacity for aerobic glycolysis. Pneumococcus III in passing to the degraded stage gained this activity, which is in accordance with the respective increase and decrease in respiratory activity of the two types. In order to appreciate the degree of the respiratory capacity of pneumococci, Finkle stated that the O_2 consumption was for Type I pneumococci thirteen times and for Type II strains thirty-four times that of the human tubercle bacillus (strain H_{37}). When compared with the oxygen consumption of animal tissues, Type II strains consumed over twenty times as much oxygen as did isolated rat kidney tissue, and almost one hundred times as much O_2 as isolated dog muscle.

A respiratory phenomenon connected with loss of virulence has been described by Sevag and Maiweg.[1258] A virulent pneumococcus on being transformed into its avirulent form consumes many times more oxygen than the parent organism, but the gain of activity is a temporary property. After a time, the avirulent variant degenerates into a form that consumes much less oxygen than either the virulent or the recently derived avirulent form. The phenomenon may be associated with the change in the structure of the enzyme responsible for carbohydrate biosynthesis during the shift from the virulent to the avirulent state and hence may be related to capsule formation. According to Sevag and Maiweg, the addition of colorless, clear, blood catalase or of a small amount of sodium pyruvate to the culture enables the organisms to carry on their

respiratory functions and to maintain their reproductive capacities and virulence for a longer period of time.

Petrie (1932)[1084] suggested one more means for the identification of R and S variants. In stab cultures in agar plates containing 5 per cent immune serum the virulent S pneumococci grew with a distinct halo about the colony when the organism and immune serum corresponded in type-specificity. The halo apparently consisted of a specific precipitate formed by the interaction of the pneumococcal polysaccharide and the precipitin in the homologous serum. The R colonies, in contrast, produced only a faint and narrow halo after a considerable period of incubation. Serum from immune horses appeared to be more suitable than serum from immune rabbits for halo production.

INTERMEDIATE FORMS

In addition to the well-known S and R forms, Klumpen (1932)[730] mentioned intermediate forms growing in colonies designated as SU and RK. In other characters, however, the strains were either true S or R forms. Klumpen recognized the *Flätterformen* described by Grumbach, and noted that the organisms comprising daughter colonies were of the smooth type.

Still other variants intermediate between the S and R forms were derived from pneumococci by Blake and Trask (1933).[130] By growing Type IS pneumococci in homologous immune serum broth, the progressive appearance and disappearance of forms differing from both S and R cocci were observed. The forms were designated as a, b, c, d, and e. Two of the intermediates, Type Ib and Type Ic, were easily stabilized in pure culture. All showed an orderly change in agglutinative reactions in homologous and heterologous immune serum, and also underwent a progressive loss of virulence for mice. Blake and Trask produced only one intermediate form from Type IIS and none from Type IIIS.

The importance of recognizing intermediate variants in the dissociative process was emphasized by Paul[1069] of Blake's laboratory. He believed that two methods of inducing degradative dissociation in S forms seemed to give rise to the different patterns of variant production. Thus, when S forms were grown in homologous antiserum they became rapidly stabilized as R forms, but when S forms were cultivated in media containing bile, the S organisms displayed a greater tendency to become stabilized as c forms. Paul showed that during the reversion of c, d, and R forms, induced by growth in anti-R or plain rabbit-serum broth, intermediate variants arose in the reverse order to that in which they appeared during the degradation of S forms. The intermediate variants tended to become stabilized as b forms, which was the usual high level to which these strains reverted by this method.

A process possibly related to that studied by Blake and Trask was reported by Eaton,[345] an associate of Blake, who described the production of stable strains of Pneumococcus which underwent rapid lysis or failed to grow at 37°. For the strains he introduced the term "phantom colony" or "P–C" variants. This P–C variation, he claimed, was a change independent of the ordinary smooth-to-rough variation. Eaton, moreover, made direct isolation of these variants from cases of human infection.

Another apparent complication in the symbols employed to identify pneumococcal variants is to be found in the recent papers by Eaton (1934–1935).[345-6] In addition to the phantom colony or P–C variants, he observed smooth variants arising in the daughter-colony dissociation of stock smooth strains after incubation on blood agar at 25°. These smooth variants, called V, and the smooth parent strain, termed N, from which the former were derived, had the same virulence for mice and did not differ in antigenic composition as determined by agglutination, agglutinin-absorption, and mouse protection tests. The smooth V strains were stable, and while they, too, formed daughter colonies they dissociated to rough forms much less readily than did the N or freshly isolated strains. The N and V strains appeared to differ in their capsular staining reactions, and in the ability to form methemo-

globin in blood. Without an actual visual comparison of these V variants with the principals and intermediates described by other authors it is impossible to assign them their proper place in the dissociation order.

Further study is required before giving an estimate of the significance of these possibly new forms, although there is already much evidence to support the concept of a polyphasic cycle in bacterial dissociation.*

REVERSAL OF DISSOCIATION

Griffith[562] was successful in reversion experiments in the animal body. Some R strains which had not entirely lost their soluble specific substance readily reverted to the S form when passed through the mouse. The author obtained smooth colonies, with restoration of virulence and original serological type characters, after making massive injections of R strains into the subcutaneous tissues of the mouse. The original change from S to R forms was accomplished by ageing the colonies on chocolate blood agar containing horse serum and by cultivation in broth to which specific immune serum had been added. The greater the concentration of immune serum, the more complete and permanent was the change to the R form.

The possibility of the reversal of the dissociation process attracted Dawson and Avery[304] who, by mouse passage, not only brought back to the S form seven or eight cultures of single-cell isolation, pure-line S strains of Types I, II, and III, but also succeeded in causing six pure-line R strains to revert to type-specific forms by growing the cultures in media containing anti-R serum.† The authors failed in a similar attempt with a Type IR culture. As in mouse passage, reversion by cultural methods was accompanied

* Rakieten[1119] believed that a peculiar organism obtained from the peritoneal fluid and heart's blood of a mouse after inoculation with a highly virulent Type II Pneumococcus was a pneumococcal variant. It was a Gram-positive bacillus, bile-soluble, agglutinated with Type II serum in a dilution of 1 to 400, and also to a slight extent with Type I serum. The organism was not pathogenic for mice. Rakieten's description of cultural development of the strain from the infected fluids raises doubt as to its true pneumococcal origin.

† Compare Soule's[1308] similar results with *Bacillus subtilis* (1927).

by acquisition of properties of the typical S form. In the experiments, reversion was invariably toward the specific type from which the R form was originally derived.*

REVERSION BY MEANS OF PNEUMOCOCCAL VACCINE

In Griffith's experiments on reversion he introduced a new principle, which later was to effect still more surprising and momentous changes in the biological character of Pneumococcus. He reported that the most certain method of producing reversion was to add to the R culture before subcutaneous injection into the mouse a dose of a heat-killed culture of a virulent strain of the same type. Reversion from R to the S form could occasionally be brought about by the simultaneous inoculation of a virulent culture of another type when the culture had been heated for only a short period to 60°, that is, a Type IIR strain reverted to its original condition when inoculated with a heated, virulent Type I culture. The Type I antigen appeared to lose the power to cause reversion more easily than the Type II antigen, the former becoming inactive after heating to 80°, whereas the latter was still effective after steaming at 100°. Griffith found, moreover, that the antigen of certain Group IV strains appeared to be closely related to that of Type II. Both were equally resistant to heat, and stimulated the reversion of R forms derived from Type II, but failed to bring about the reversion of the RI strain to its S form.

Transformation of Type

More surprising and important was the successful transformation by the method of an R strain derived from one specific type into the S form of the same type as that of the heated culture. The S form of Type I was evolved from the R form of Type II Pneumococcus, and the S form of Type II from an RI organism.

* Alloway (1932)[8] cited Kelley as having discovered that normal hog serum was rich in these anti-R bodies and could be substituted for anti-R serum in activating the reversion process.

From the RI variant and from the R forms of Type II, were derived the clear, mucinous colonies of Type III. The newly developed Type III strains were of relatively low virulence and frequently remained localized at the site of subcutaneous inoculation. A still wider shift which Griffith effected was that of a Group IVR strain to virulent strains belonging to Types I and II. The injection of large doses of heated cultures of R pneumococci along with small amounts of living R strains never caused a transformation of type and only rarely produced a reversion of the R form of Type II to its S form. Griffith, therefore, along with his success in changing R variants back to the original S forms with accession of virulence and specific type characters, was the first to accomplish a true transformation of one pneumococcal type into another.

To degrade a pneumococcus *in vitro* to a form devoid of its original type characters and then to exalt it to its original condition was an achievement that we had come to expect, but transforming a degenerated or dissociated culture into another form possessing entirely different type characters was a somewhat amazing performance. Even remembering the theories of earlier investigators with their claims of species mutations, and discounting possible errors in their experiments, this discovery had not been anticipated. It was, for the first time, to supply a theoretical explanation for the many baffling problems encountered in the study of the spread and the invasiveness of pneumococci, and of the clinical pathology of pneumococcal infections, not to mention the broader bearing on the many riddles of microbiology.

Neufeld and Levinthal[994] also were able to reproduce Griffith's transformation phenomena, but by another procedure. They first dissociated virulent, type-specific strains by growing the organisms in broth containing sterile animal organs (spleen, heart, kidney, and liver of rabbits). The degraded R variants were then injected subcutaneously into mice with killed S pneumococci. Neufeld and Levinthal thus converted an avirulent Type IR pneumococcus into a virulent Type IS organism, and with the addition of

a killed Type IIS strain obtained a typical IIS pneumococcus. Not all the R variants could be reverted.

Somewhat less success in this respect attended the efforts of Reimann.[1129] The R strains evolved by immune serum-broth cultivation were as a rule irreversible, only one of many strains passing back to the S form of Type I or over to Type III, the reversion depending upon the type of the heated culture used. No transformations to Type II occurred, although in one instance it appeared that a heated IIS culture induced the reversion of the R strain to the Type IS prototype. Reimann obtained positive reversions of typical R forms from pneumococci of Types I or II when he inoculated the R strains subcutaneously into mice with heated S cultures of Types I, II, and III. The living IR culture plus heated Type IIS vaccine gave Type IIS pneumococci; IIR became IS or IIIS, depending upon the type of heated culture used. Both Types IR and IIR, when inoculated with heated cultures of homologous type S forms, frequently reverted to the respective prototypes. These seemingly bizarre biological changes were, therefore, becoming a routine laboratory performance.

Baurhenn's[93] efforts at reversion (1932) were more fruitful than Reimann's. By subculturing R strains with homologous and heterologous vaccines consisting of heat-killed cultures, he changed the R strains into their original S forms and to the S form of a different type. Baurhenn inclined to Griffith's view that all pneumococcal types possess a common basic form. The basic form, under the stimulation of the activating principle, responds by acquiring the properties of the activator. Baurhenn claimed to be the first to have produced transformation within Group X (Group IV) as well as the transformation of a fixed type (I, II, or III) into a specific type of Group X. This feat is, of course, entirely possible, and from what we already know of the phenomena of transformation, there is no reason to doubt that similar changes may occur in the case of all the known types of pneumococci.

Dawson (1928)[299] confirmed and expanded Griffith's observa-

tions. He found that type-specific S pneumococci could be transformed from one specific S type into another specific S type through the intermediate stage of the R form; that R forms of pneumococci, derived from any specific S type, might be transformed into S organisms of other specific types by injecting mice subcutaneously with small amounts of living R strains together with heated vaccines of heterologous S cultures. The S vaccines could be heated for fifteen minutes between 60° and 80° and still remain effective in causing R forms derived from heterologous S types to revert to the type of the vaccine; S vaccines heated fifteen minutes at temperatures between 80° and 100° were not active in causing R variants derived from heterologous S types to revert to the type of the vaccine; S vaccines heated between 80° and 100° could cause Type IIR and Type IIIR variants to revert to the original S type; S vaccines of any type, including Type I, heated for fifteen minutes at 80° to 100° would no longer cause Type IR strains to revert to their original S type; S vaccines heated for periods as long as two hours at 60° were effective in causing R forms derived from heterologous types to revert to the type of the vaccine employed. Dawson successfully converted a single-cell R strain derived from a Type IIIS pneumococcus into a Type IIIS, a Type IS, and a Group IVS organism. On the other hand, every attempt to produce transformation of type *in vivo* failed.

TRANSFORMATION BY VACCINE AND ANIMAL INOCULATION

In 1930, Dawson and Sia[305] announced the transformation of a Type IIR into a Type IIIS pneumococcus. The conditions necessary for the reversal were minimal amounts of the R culture, the addition of the heated activating culture, incubation for longer than the conventional period, and the inclusion of small amounts of anti-R serum and of blood broth. When the activating organisms were heated for fifteen minutes at 100°, they lost their capacity for inducing transformation, although suspensions heated for four hours at 60° or for fifteen minutes at 80° were still effective.

Filtrates of vigorously growing cultures and of heat-killed suspensions of S organisms were inactive, as also were suspensions of S organisms disrupted by freezing and thawing, with subsequent heating for fifteen minutes at 60°. But when suspensions of S organisms were first killed by heating for fifteen minutes at 60° and then frozen and thawed, they were highly effective. In a more detailed communication, the authors gave the additional information that transformation of type could be induced by the use of small amounts of S vaccine, and that while the transformative process was brought about most readily by employing anti-R serum in the culture medium, it might be accomplished without the presence of the serum.

Transformation of one S form to the S form of a different type without any apparent development of intermediate stages was described by Dawson and Warbasse[306] in 1931. The original culture was a virulent, single-cell isolation of Type II Pneumococcus. One drop of a 10^{-6} dilution of the culture was seeded into a medium containing homologous immune serum together with large quantities of Type III pneumococcal vaccine. The cultures were incubated at 37°, and at the end of forty-eight hours streaked plates showed, in the majority of instances, numerous Type III with some Type IIS colonies. No R colonies were observed. From the experiment Dawson and Warbasse inferred that a type-specific S pneumococcus can be transformed into other type-specific S pneumococci by growth in homologous immune serum in the presence of heterologous vaccine. Although the conditions of the experiment were unfavorable to the development of R forms, the authors thought it was probable that the organism nevertheless passed through this intermediate stage during the transformation.

In 1931, Sia and Dawson[1272] reported that R cultures possessing slight degrees of R stability were most suitable for transformation experiments *in vitro*. The authors also sought a soluble principle in cultures subjected to the action of bacterial enzymes liberated in old broth cultures and during mechanical disruption of

young bacterial cells. Trials with the solutions, with the supernatant fluid from an S vaccine, the filtrate from an S vaccine, purified soluble specific substance, and the filtrates of actively growing S cultures, all gave negative results.

ISOLATION OF THE TRANSFORMATIVE PRINCIPLE

Alloway (1932)[8] evidently was more successful than his predecessors in obtaining the transformative principle from the pneumococcal cell. With filtered extracts of virulent S strains of Types I and III he converted a Type IIR strain into S organisms of the same specific type as that of the cells extracted. The author stated that the constituents of the extract supplied an activating stimulus of a specific nature in that the R pneumococci acquired the capacity of elaborating the capsular material peculiar to the organisms extracted.

In the next study (1933), Alloway[9] prepared active and specific extracts by dissolving S pneumococci in sodium desoxycholate solution. These cell-free extracts were as potent as the intact cocci in causing R forms to assume new type-specific characters. With an extract of Type III Pneumococcus he was able to convert a Type IIR variant almost regularly and abruptly into the smooth form of Type III. Alloway then purified the extracts by removing a considerable amount of inert material by charcoal adsorption and reprecipitation of the adsorbed extracts with alcohol or acetone. The stimulating principle passed through Berkefeld filters without loss of strength if the reaction of the solution was alkaline. The substance was resistant to heating at 60° for thirty minutes but was appreciably affected at temperatures of 80° or over. The purified extracts apparently had suffered no loss of potency and caused a more prompt transformation than did the original solutions. An unexplained observation was the fact that in no instance could the transformation be effected without the addition to the culture-extract mixture of blood serum or of ascitic or pleural fluid.

Photograph by Louis Schmidt Courtesy of the Rockefeller Institute for Medical Research

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Dawson Classification

A dissociation form, other than the S and R forms, was described in 1934 by Dawson.[803] It appeared to be a mucoid variant of Pneumococcus and was strikingly different from the two main, accepted variants. Dawson intimated that the terminology of bacterial dissociation should be changed to include the M form.

In later communications (1934), Dawson[802-3] gave many more details of the several stages of pneumococcal dissociation. He showed, first, that the change from the typical, virulent form to the degraded variant was not a simple direct S → R conversion, but that the dissociative process consisted of several phases. In this cycle there were three outstanding stages represented by distinct difference in colony appearance and morphology, and here he departed from the orthodox concept of the S and R forms.

At first reading, Dawson's discussion and proposals are a little bewildering. He makes the apparently radical suggestion that the old designations smooth and rough be changed and the term "mucoid" be introduced into the terminology as it applies to Pneumococcus; thus, S would become M; R would become S; and the new form would be R. His revelation of the intricacies of the dissociative phenomena and the proposed change in terminology are apt to cause some confusion in minds accustomed to the accepted order of dissociative nomenclature. But an unprejudiced and painstaking study of the facts and his recommendations serves to dispel some of the doubts raised in a cursory reading of the text. Dawson's contentions were founded on the appearance of a new variant during the cultivation of an R form of Pneumococcus originally derived from a Type IIS culture. When this strain was diluted, thinly streaked on blood-agar plates, and incubated several days at 37°, many of the colonies showed evidence of a variety of secondary growths. The following is taken from Dawson's description of the S → R transformation.

For convenience the evolution of the R variant may be described in several stages although it is emphasized that the process is both gradual

and continuous. In the first stage, which may suitably be termed R^1, the colonies present more or less the general appearance of smooth (old terminology, "rough") colonies but the surface is more coarsely stippled. The constituent organisms are more or less typical pneumococci showing a tendency to staphylococcal grouping and occasional swollen or club forms may be seen. In the second stage, R^2, the colonies present a still rougher appearance and the outline may appear slightly irregular. This irregularity frequently becomes quite pronounced after several days' growth. The bacteria in this stage are much more pleomorphic and are frequently elongated in an extreme lanceolate manner. They still retain Gram's stain. In the third stage, R^3, the surface of the colony becomes exceedingly rough and the margin quite irregular. The contour of such colonies still remains convex but less so than the original S form (old terminology, R form). The organisms constituting such colonies present a bizarre morphological picture. Pointed diphtheroid elements arranged in a fashion suggesting broken twigs may be observed, with scattered long, bizarre, rod forms which are partially Gram-positive and partially Gram-negative. At this stage of development the morphological picture can scarcely be recognized as that of pneumococcus. The fourth stage, R^4, can only be defined with some difficulty. It would appear that the growth is now in a stage of considerable flux and several types of colonies and morphological elements may be produced. Some of the colonies present an appearance similar to that just described while others resemble more closely the pure R form (R^n).

The morphology of the organisms in the R^4 stage is difficult to describe because of their extreme pleomorphism. In addition to coarse and irregular coccal forms there may appear elongated Gram-negative rod-like structures exhibiting irregular Gram-positive areas. A great variety of other morphological elements may also be present.

Dawson described the R → S change in which intermediate forms of the type seen in the S → R change were not observed, and then gave a detailed description of the biological characters of this new variant. From the description a few of the more important data may be selected. The organism was bile-soluble, of low virulence for mice, agglutinated in normal saline solution, and failed to elaborate soluble specific substance. The variant was not peculiar to the

Type IIR strain, and single organisms of one individual strain also possessed the capacity to dissociate into the new form.

Dawson then pointed out certain discrepancies in the characteristic features of the S and R forms as described by Griffith for Pneumococcus and those described by Arkwright for the colon-typhoid-dysentery group, and which have been accepted by the majority of bacteriologists as the chief distinguishing features of the smooth and rough forms of many bacterial species. He further drew attention to the fact that certain attributes of Arkwright's S and R forms do not appear in Pneumococcus while other new distinctions did not have a place in Arkwright's original descriptions. Dawson has portrayed these differences in terminology in a diagram which, although as yet unpublished, was kindly loaned to the authors.

	COLON, TYPHOID, DYSENTERY		PNEUMOCOCCUS	
	ARKWRIGHT	OTHER OBSERVERS	GRIFFITHS	DAWSON
1	NOT DESCRIBED	M	S	M
2	S	S	R	S
3	R	R	NOT DESCRIBED	R

Courtesy of Dr. M. A. Dawson

RELATIONSHIPS OF MUCOID, SMOOTH, AND ROUGH COLONIES OF BACILLI OF THE COLON-TYPHOID-DYSENTERY GROUP AND PNEUMOCOCCUS

When Dawson compared the salient characters of the three pneumococcal variants (S, R, and the new M form) with the mucoid, smooth, and rough forms of members of the colon-typhoid-dysentery group and the smooth and the two rough forms of the Friedländer bacillus, the inconsistency in the use of the terms smooth and rough became convincingly apparent. On a basis of colony appearance, morphology, growth in plain broth, stability in salt solution, and of virulence and type-specificity, the smooth form of Pneumococcus and of Friedländer's bacillus conforms to the mucoid form of members of the colon-typhoid-dysentery group; the rough form of Pneumococcus and the R_1 form of Friedländer are similar to the smooth form of bacilli of the enteric group; while Dawson's new variant and Julianelle's R_2 form of the Friedländer bacillus agree with the rough form of the colon-typhoid-dysentery bacilli.

In order, therefore, to bring these terms in agreement, to conform—with an addition—to the designations of Arkwright, and to establish a uniform and logical terminology for the dissociants of all bacterial species, Dawson would change the terms now used for the variants of pneumococci as follows: Mucoid or M would replace the present smooth or S; smooth would be substituted for the former rough (R_1 form of Friedländer bacilli); while rough or R would be applied to the new variant described by Dawson and the R_2 form of Friedländer's bacillus.

There is no doubt that such a reversal of the accepted terms would cause confusion and meet with opposition. It cannot be denied that this change would be especially disturbing to the present correlation between the classification of dissociation forms and immunological behavior, but that does not necessarily preclude the possibility of a new and perhaps a deeper insight into the parallelism between the phenomena of variation and antigenic specificity. This proposed change recalls the confusion that followed the revision of the designations of blood groups, but that change has not only been endured but the new terms are now generally accepted as

useful and logical. There is much to be said both for and against Dawson's proposal and so it may be permissible to turn to one who speaks with authority on this important subject of bacterial dissociation. Hadley's opinion expressed in a letter written in 1933 to Dawson was in part:*

> Making a decision regarding the proper course to pursue in changing the nomenclature now employed for designating the phases of the pneumococcus, in favor of the symbolization which your studies thus far seem unquestionably to justify, might easily depend on how fully an investigator has in mind the details of dissociative variation as a phenomenon observable in all bacterial species, and how clearly he can perceive the parallel trends in such variations,—as opposed to a limited outlook on the one species in which he may be especially interested.
>
> If bacteriology were limited to the study of a few species, or to the Pneumococcus, it would make little difference what the observed phases were called, because no generalizations would be involved, and the phase symbols would possess no significance for bacteriology as a whole. A, B and C, or X, Y and Z would serve the purpose. . . .
>
> The desirability of adjusting the difficulty in the Pneumococcus situation, and of doing it without delay, is the more to be recommended in view of the increasingly wide recognition that the same or analogous phases exist in numerous other species. The facts are now becoming so extensive and well grounded that they are offering, for the first time in the history of bacteriology, a basis for the formulation of general laws; and for making possible a certain kind of "predictability," as I have perhaps already demonstrated to you. To this extent pure bacteriology is beginning to take on the aspects of a real science—a compliment which (to my mind) it has scarcely been appropriate to offer in the past.
>
> To facilitate this highly gratifying trend it stands to reason that all who work with the problems of variation should keep in mind the dual significance of their results, and make possible a correlation of their own results with those of others; also to make quick and decisive corrections when such are clearly in order. To label as a smooth a Pneumococcus phase that is demonstrated to be a mucoid, or to label as a rough a phase that is clearly a smooth, may do little harm to those

* The authors appreciate the courtesy of Doctors Hadley and Dawson in granting permission to include portions of this letter here.

whose work lies chiefly in this species. But such a continued policy can only render increasingly difficult important comparisons with other species, and work havoc with the interests of those who are seriously attempting to discern some law and order in the affairs of the bacteria. Further advance in this direction can take place, according to my view, only if bacteriologists become sufficiently keen to recognize the true nature of the phases they employ, and sufficiently independent to "call a spade a spade," whenever recognized as such, regardless of politics, tradition or social etiquette. . . .

It might also be in the back of your mind that the splendid work of some of your associates on the chemical aspects of dissociation would suffer from any change in terminology made at this late date. I am absolutely convinced to the contrary. In reality I believe that the incentive to extensions of their results to many other bacterial species would be a direct and immediate outcome, through establishing a recognition of the most appropriate culture phase to be employed in such studies. . . .

It is therefore my opinion that a frank recognition of the present incongruities of the situation will not detract from, but facilitate in wide measure, researches in the important field opened up years ago by Drs. Avery, Dochez, Heidelberger and their collaborators.

Dawson believed that before making such a radical change in the accepted terminology of pneumococcal variants it would be well to ascertain if similar variants could be demonstrated in *Streptococcus haemolyticus*. From the latest study by Dawson,[303] it would seem that he succeeded in dissociating that organism into three main variants, which in their manner of colony formation and in morphology correspond closely with the three main variants of Pneumococcus. The mucoid and smooth forms appeared and, by cultivation of the streptococci on blood agar and by repeatedly picking and transplanting material from the roughest marginal areas, Dawson was able to develop the extremely rough type of colony which he had obtained with pneumococci, representing the R variant.

As Dawson said, "evidence is rapidly accumulating to show that the phenomenon of bacterial variation in a wide variety of bacterial species fits into a more or less orderly pattern." This pattern, besides fitting bacilli of the colon-typhoid-dysentery group, the types of *B. friedländeri*, and probably the streptococci, would bring order in the arrangement of the many variants of pneumococci that have been described under a wide diversity of terms. Thus, the modifications A, B, and C of Schnitzer and Berger, Blake and Trask's intermediates Type I a, b, c, d, and e, Wadsworth and Sickles' atypical strains, Reimann's daughter-colony variants, the "wall" type of Buerger, the *Flätterformen* of Grumbach, possibly the P–C or phantom colonies and the smooth N and the smooth V types of Eaton, the variants of Kimura, Sukneff, and Meyer, the atypical rough forms from budding colonies reported by Paul, the SU and RK dissociants of Klumpen, and of course the R and S forms of Griffith, and the new variant of Dawson might conceivably be arranged in accordance with the general pattern and would all either fall into the chief places designated by Dawson's M, S, and R or into the spaces between these predominating forms.

The scheme of Dawson, therefore, revolutionary as it may seem, merits further consideration and should be subjected to additional experimental trial before it is rejected or finally accepted.

These discoveries concerning the variability of Pneumococcus are full of new meaning to the bacteriologist, biochemist, immunologist, and particularly to the physiologist. They prove that Pneumococcus has the potential ability to synthesize simple sugars into diverse, complex, and highly individual polysaccharides. When the conditions of the surroundings are entirely favorable, this metabolic process operates uniformly. The end products are always of the same molecular composition and configuration, and are highly distinctive of a given serological and biochemical type. When, however, the forces of the environment are inimical, the function of carbohydrate synthesis is retarded, the cell produces less and less of the distinguishing capsular polysaccharide, and the cocci lose their capsule, virulence, and strict racial identity. If the

unfavorable conditions continue, this particular metabolic activity ceases or is suppressed and the organism degenerates into a harmless coccus, devoid of any specialized earmarks—a sort of bacterial maverick. If the exposure to these untoward conditions is sufficiently protracted, the function is apparently permanently lost, but if the exposure ceases before this stage is reached, the cell retains the latent power to elaborate its original, individual capsular carbohydrate, and all that is needed to revive this power is the restoration of a satisfactory environment—either in culture or in an animal—or else the activation that comes from an encounter with immune bodies specific for its own degraded form. Living under such conditions the type-less coccus gradually returns to its former distinctive state.

These discoveries, moreover, have disclosed another and astonishing activity of the organism. When stimulated by some unknown constituent of fully functioning pneumococcal cells, this latent metabolic function of the degenerated coccus develops a new property, and instead of building up capsular carbohydrates of the former kind, the degraded cell now synthesizes polysaccharides of the same chemical constitution and specific type as those of the strains supplying the activating stimulus. The once degraded organism becomes then a virulent pneumococcus, but with all the specialized characters of its foster strain. Having lost its original features it regains a new type identity.

The cycle of degradation, regeneration, and type transformation presents so many fascinating phases that one is strongly tempted to speculate on the various factors concerned in this extraordinary evolution. The basic ability to elaborate these various specific capsular carbohydrates is always ready to respond to appropriate stimulation unless the cells have gone too far down the path of degradation, and is evidently common to all pneumococci. The direction which the transformation takes is determined wholly by the nature of the stimulus, and it is the identity of this factor which still remains unrevealed to us. It apparently exists only in

cells exercising all their special functions, and seems to be a normal constituent of the cell and not a product of katabolic processes.

Whether such transformations ever take place in the animal body, in health or in disease, and if they do what causes bring them about, together with the yet broader problems of the origin of various types and the influences which established their different biological identities, are all questions that are attracting investigators in this branch of science. Whether this fundamental function of Pneumococcus can be so perverted as to bring about the transmutation of this organism into one of a different species is a problem which has been attacked in a more general way.

Transmutation of Species

The mutability of members of the bacterial tribe *Streptococcaceae* has long been a moot question. From time to time there have appeared reports of the change of a pneumococcus into a streptococcus, and even of a swing through the whole cycle from virulent Pneumococcus to *Streptococcus viridans* to *Streptococcus haemolyticus* and back to Pneumococcus. But, in these later days of refined bacteriological and immunological technique one has been inclined to look somewhat askance at such claims. The idea has, however, persisted, and what was looked upon as a mere notion is now becoming so much more than a hypothesis that there are those who would accept this metamorphosis as an accomplished fact.

There is no call to recite at any length the accounts of the early experiments. Some were based on crude, faulty methods which always raise doubts as to the purity of the cultures the pioneers studied. Disregarding claims resting solely upon morphological or cultural phenomena, it is better to confine the discussion to reports, with a few exceptions of historical interest, that have been published since the development of modern bacteriological and serological technique. In 1891, Kruse and Pansini,[763] by transplanting forty-six strains of pneumococci on media unfavorable to growth, developed eighty-four varieties that exhibited differences

in character all the way from typical *Diplococcus lanceolatus* to *Streptococcus pyogenes*. The authors stated that the relation of pneumococci to streptococci was clearly evident, and that the origin of these bacterial species was a single, probably saprophytic, streptococcal form.

There the matter rested until 1907, when Buerger and Rytten-berg[169] described an organism isolated from a case of puerperal pneumococcemia which, although originally failing to ferment inulin and exhibiting streptococcal characters, developed into a typical pneumococcus after animal passage. The observation led the authors to study a number of cultures isolated from human exudates and blood, and with these strains they observed characters typical of streptococci which, however, gave way to pneumococcal characters after propagation in mice. Buerger and Rytten-berg concluded:

> The tendency of pneumococci of the streptococcus cultural type as well as those which have been converted to the normal variety, seems to be toward a gradual degeneration which manifests itself in the assumption of permanent streptococcic features. Such organisms can then no longer be differentiated from streptococci.

In 1909, Rosenow[1162] made the statement that strains of *Streptococcus viridans* isolated chiefly from the blood in cases of subacute endocarditis and obtained also from the throat and other sources might by animal passage take on the properties of typical pneumococci, and hence designated them as "modified pneumococci." Rosenow also claimed that during a study of autolysis of pneumococci in salt solution and of the effect of sodium oleate and bile on virulent pneumococci he had observed transformation of the strains into hemolytic streptococci. The statement appears to be conservative when compared to Rosenow's[1170] description in 1914 of the various transmutations accomplished within the Streptococcus-Pneumococcus group. He told of converting by cultural methods twenty-one strains originally isolated as hemolytic streptococci from cases of erysipelas, scarlet fever, puerperal sepsis, arthritis, and tonsillitis, as well as from cows' milk, into *Streptococcus viridans;* of changing three similar strains into *S. viridans* and typical pneumococci, and one into *Streptococcus mucosus* as well. Seventeen strains isolated as *S. viridans*, chiefly from the blood and tonsils in cases of chronic infectious endocarditis, and two strains from cows' milk were converted into pneumococci while two of the strains became *S. mucosus*. Ten of the *viridans* cultures were made to take on the cultural and morphological characters of hemolytic streptococci, in two of which the pathogenic powers were shown to be those of hemolytic streptococci, while one strain was converted into a hemolytic streptococcus, into *S. viridans*, and then into a pneumococcus.

Rosenow claimed to have converted into hemolytic streptococci eleven strains isolated as pneumococci from sputum, blood, and the lung in pneumonia and from human empyema fluids and Cole's Type I and II strains, while seven cultures took on the features of *S. viridans*. The streptococci derived by animal passage from three of the pneumococcal strains were alleged to acquire all the essential features of the streptococci of rheumatism, and two organisms were said to have been converted into hemolytic streptococci, the streptococci of rheumatism, *S. viridans*, and back again into Pneumococcus.

Rosenow further alleged that the transformation of some of these strains, checked in a few instances by single-cell isolations, was found to be complete by every test known. The tests included the study of morphological features, the demonstration of capsules, and observations on fermentative powers, solubility in bile and in saline solution, the behavior toward the respective broth-culture filtrates (Marmorek's test), the specific immunological response as manifested by the appearance of opsonin and agglutinin in antistreptococcic and antipneumococcic serum, and the more or less specific pathogenic powers of the various organisms.

In summary Rosenow wrote:

The changes observed have frequently the characteristics of true mutations because they appear suddenly, under conditions more or less obscure and because the newly acquired properties persist unless the organisms are again placed under special conditions. A pre-mutational stage seems to be necessary because the same strain will not yield mutants when placed under what seem to be identical conditions at different times. The underlying conditions which tend most to call forth changes are, first, favorable conditions for luxuriant growth and then unfavorable conditions—under stress and strain. This seems to call forth new or latent energies which were previously not manifest and which now have gained the ascendency and tend to persist. This may hold true *in vivo* also. This fact makes it difficult to obtain mutations outside of the body with highly virulent strains, because they die before there is opportunity for the organisms to adjust themselves to the new conditions. It explains why injection into cavities makes for greater changes than intravenous injections of moderately virulent organisms. Apparent mutations in animals have been observed almost exclusively in closed cavities, such as joints and pericardium, and here mostly when the tissues of the host were gradually getting the upper hand and the organisms were being destroyed. The mutations *in vitro* may be spoken of as "retrogressive" and those in animals as "progressive" because evidences of a vigorous vegetative life are diminished whereas in the latter they are usually increased.

The results and conclusions of Rosenow have been transcribed in some detail because they represented such a wide departure from established belief. The announcement was greeted with much skepticism. Such sudden and wide shifts from one to another supposedly fixed species appeared to violate biological laws, and it seemed that some artifact must have been responsible for the remarkable transformations. Nowhere in the literature, with the exceptions to be described, have references been found which duplicate or substantiate Rosenow's results.

Wolff (1923),[1584] in a long theoretical discussion of pneumococcal mutation, suggested that the members of the large tribe *Streptococcaceae*, from pure saprophytes to true parasites, in spite of

all differences, were really linked together. He claimed to have obtained mutations by gradual adaptations of the organisms to the host. The attempts met with many failures which were explained by saying that if the organism was too weak it died in the host, and if too virulent it killed the host before any accommodation had taken place. Wolff asserted, however, that in three cases he had transformed *Streptococcus viridans* from *endocarditis lenta* into Pneumococcus. The organism became bile-soluble, optochin-sensitive, developed a capsule, fermented inulin, and was lethal for mice. Evidence of bacterial mutations of any kind coming solely from *in vivo* experiments is to be weighed with caution.

Neufeld[979] in a discussion already cited on microbic variability, recalled an observation he had made ten years previously on the original "Pneumococcus I" of Neufeld and Haendel, which had been preserved by drying and storage in a dessicator. One mouse inoculated with the culture produced a strain growing in chains, insoluble in bile, but virulent for mice, and with all typical streptococcal properties. At first Neufeld thought he had made a mistake in the material he injected, but a similar experience of Schiemann's convinced him that a mutation had actually taken place. Coming from anyone less eminent than Neufeld, this single, isolated observation would be disregarded.

In the following year, Morgenroth, Schnitzer, and Berger[929] announced that by special methods they had been able with regularity to transform pneumococci into streptococci.* Their medium contained dead yeast cells or animal charcoal which had adsorbed optochin. The altered strains became insoluble in sodium taurocholate, were avirulent for mice, and were resistant to optochin. Modification A represented the first stage in the transmutation. The organisms retained the majority of their pneumococcal characters, but were more resistant to optochin and more sensitive to

* Stanka[1312] called attention to the fact that these authors had omitted mention of similar work published by Elschnig and Ulbrich, and by Kraupa from the German Eye Clinic at Prague.

rivanol than were cocci of the original stock. In Modification B, the colonies, made up of A after growing in optochin, resembled those of *S. viridans*. The cultures contained long chains of round cocci, which were bile-insoluble and were very resistant to the pneumococcidal action of optochin. Modification C developed after further growth on artificial media or in animals, and occasionally after growth in an optochin medium. The C variants corresponded to *Streptococcus haemolyticus*, they produced more or less hemolysis on blood agar, were bile-insoluble and optochin-fast, but sensitive to rivanol. The progressive changes did not always take place or follow the A-B-C sequence. In twenty-nine experiments with fifteen strains, twenty-two trials produced modifications A and B, and of these strains seven were transformed into modification C.

Berger and Englemann[100] continued similar mutation experiments and alleged to have demonstrated Modification A in five specimens of sputum and one of pleural exudate obtained from pneumonia patients before the disappearance of fever. The strains were then converted into Modification B by allowing a fairly high concentration of optochin to act upon them. Berger and Englemann also claimed that the complete transformation could take place in the human organism. To support the claim the authors described the development of glistening Type III colonies along with a few strongly hemolytic streptococcal colonies on a blood-agar plate upon which pneumonic sputum had been spread. The organism, after the first mouse passage and three culture generations, developed into a green streptococcus; after a second direct mouse passage both pneumococci and hemolytic streptococci appeared, the latter partly reverting to Pneumococcus after two culture generations. The original hemolytic streptococci after three culture generations became green streptococci and after four culture generations reverted to pneumococci. This cycle, like Rosenow's, seems almost too rapid and direct to be credible.

In another communication, Berger and Jakob (1925)[102] returned

to earlier experiments on the development of B and C modifications. During animal passage of short duration, the changes were less marked, since the authors reported only a transient loss of virulence. Berger and Englemann[101] in the next year reported the mutation of a strain of Type III Pneumococcus through the intermediary A modification to a green streptococcus. As in their former experiments, the agents necessary for the transformation were dry yeast-broth and serum-broth containing one five-thousandth part optochin. Wirth[1523] believed that *Streptococcus mucosus* represented a mutation from Pneumococcus, but he failed in his attempts to prove it.

In yet another paper Berger with Silberstein[103] described the inulin-fermentative power of the variants. The results are difficult to understand. Of ten strains of pneumococci, four showed merely a reddening of the inulin medium without coagulation, while two failed to display any action on inulin. The authors then classed the latter strains when tested with optochin with Modification A. Of the cultures of Modification B, obtained from pneumococci, but otherwise behaving as green streptococci, two retained the ability to ferment the carbohydrate. The strains were comparable in their behavior toward inulin to some thirty *viridans* strains. Of the latter, five exhibited a marked action on inulin, and four others gave slightly positive reactions.

Reimann,[1127] repeating the experiments of Morgenroth, Schnitzer, and Berger, claimed, however, that the R cultures so derived were still pneumococci, since the strains were bile-soluble and autolyzed with greater readiness than did streptococci. The immunological reactions of the variant pneumococci derived by Morgenroth's method, moreover, were identical with those of R pneumococci derived by various other means. When one considers the atypical action of the Berger strains on inulin and the author's omission of serological tests, one is inclined to accept Reimann's interpretation as the correct one.

Heim and Schlirf,[633] likewise, were unable to verify the work of

Morgenroth and his collaborators, yet Silberstein,[1286] who quoted these authors, by the aid of optochin *in vitro*, claimed to have experienced no difficulty in carrying a Group IV pneumococcus through the successive stages of Modification B (green Streptococcus) to Modification C (virulent hemolytic Streptococcus) and then from this form to a Type I pneumococcus of low virulence. Paul[1070] was another to join the newer school which believed that the gap between pneumococci and streptococci could be bridged by these methods. He produced bile-insoluble dissociants and to him they appeared to be indistinguishable from certain strains of *Streptococcus viridans*.

Görander (1930)[542] also stated that he had transmuted cultures of *Streptococcus viridans* into bacterial forms that in every respect were identical with the type-specific pneumococci of human origin, except that the strains were not agglutinated by antipneumococcic serum. The defect would seem to be a vital one. The cultural changes were accomplished by repeated cultivation on blood agar and by short mouse passages. According to Görander, after the third short (four-hour) mouse passage, hemolytic streptococci appeared. Following five twenty-four hour incubation periods in mice, the organisms resembled pneumococci. The variants had capsules, were soluble in sodium taurocholate, and were moderately virulent for mice. The pneumococci so obtained, after repeated growth of this passage culture in artificial media (alternating blood agar and broth), were retransformed "into a bacterium of perfect *Streptococcus viridans* type."

Görander claimed further to have transformed *Streptococcus viridans* and Type I and Type II pneumococci into forms which he considered to be their original state, "since they were absolutely equal culturally, biologically and serologically in all respects." The homologous antiserum agglutinated both strains, and "the bacteria absorbed not only their homologous but also heterologous agglutinins from both sera." Görander's further conclusions were so heterodox that they are quoted here:

> Finally single cell cultures originating in their time from a single cell of a pneumococcus have been examined with regard to the degree of dissimilarity which such cultures can eventually show. These experiments gave the result that two pneumococcus cultures, obtained from the same cell, can show much greater dissimilarities than two cultures obtained one from a typical *Streptococcus viridans* and the other from a typical *Streptococcus lanceolatus*. . . . Thus *Streptococcus viridans* and *Pneumococcus lanceolatus* seem to be different forms of the same bacterium, and the specific agglutinability, which *Pneumococcus lanceolatus* shows when grown from the human body and which has been taken as a base for the so-called type classification, is only an occasional character.

Summary

Virulent pneumococci of all the known serological types, upon encountering unfavorable physical, nutritional, or other biochemical conditions during growth or storage, undergo marked changes in virulence, in ability to elaborate capsules, in colony development, and in their immunological characters. In studies on the dissociation phenomena displayed by pneumococci, a great variety of aberrant coccal forms have been observed which are intermediate between the typical, virulent form and the thoroughly degraded, atypical form. So many variants with such a diversity of biological characters have been described and so many designations have been given to the intermediate forms, that it is difficult to gain a clear conception of the significance of the many phases of pneumococcal dissociation. In order to bring order out of this chaos and to make the nomenclature applied to pneumococci uniform with that employed in naming the variants occurring in the case of other bacterial species, it has been proposed to change the terminology now in use. Mucoid or M would replace the present smooth or S; smooth would be substituted for rough; while rough or R would apply to a recently discovered variant. Whatever the fate of the proposal, the alterations in character which may be induced in pneumococci by appropriate means constitute one of the most important features in the biology of the species.

During the dissociative process antigenic action may vary from one of strict type-specificity to one merely of the broader species-specificity. Degraded forms may, if the degenerative process has not been complete, regain all their original morphological, cultural, and immunological characters. Regeneration can be accomplished by rejuvenating the strain by passage through a susceptible animal, by cultivation in media containing an antiserum produced by immunization with the degraded forms, or through the stimulus afforded by heat-killed virulent cultures of an homologous type. Degraded variants, moreover, can by the action of devitalized, virulent pneumococci, actually be transformed into pneumococci of types entirely different from those from which the variants were derived and identical with those of the cultures stimulating transformation. The nature of this transformative or mutative principle is still unknown, but it is probable that it is a constituent of the pneumococcal cell and not an extracellular product of its metabolism.

The broader transmutation of Pneumococcus into Streptococcus and of Streptococcus into Pneumococcus has been advanced as a biological possibility. Experiments have been described in which it was alleged that this transmutation took place. Not only has it been claimed that both virulent and degraded pneumococci were converted into avirulent *Streptococcus viridans*, but the organisms were said to have become virulent hemolytic streptococci, while the streptococcal forms have been further changed into pneumococci. Such radical departures from established theory require the closest scrutiny of the evidence advanced and of the accumulation of new and confirmatory facts before they can be accepted.