THE INTERCONVERTIBILITY OF "R" AND "S" FORMS OF PNEUMOCOCCUS.

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The terms "R" ("rough") and "S" ("smooth"), to designate two "variants" of the same bacterial species, were first employed by Arkwright (1). His observations were made on members of the colon-typhoid-dysentery group but were soon extended to many other organisms. Griffith (2) was the first to recognize two corresponding "variants" of Pneumococcus, and his findings were confirmed and extended by Reimann (3) and Amoss (4). The distinguishing features of the two forms of Pneumococcus may be summarized as follows: "S" forms are virulent; they produce the specific soluble substance, upon which type specificity depends (5); and they form colonies which have a smooth surface when examined by reflected light. "R" forms are avirulent; they do not produce the specific soluble substance; and they form colonies which have a rough surface when similarly examined.

Since the recognition of these two forms, much interest has centered in the question of their interchangeability. It is obvious that this subject, as Hadley (6) points out, has considerable significance, not only in the problem of epidemiology, but also in the interpretation of bacterial mutation.

It is proposed to review here the question of the interconvertibility of the two forms only in so far as it applies to Pneumococcus.

As early as 1891, Roger (7) reported that Pneumococcus, when grown in the sera of inoculated animals, became attenuated in virulence. Issaeff (8), in 1893, however, showed that this apparent loss of virulence was due to the protective action of the serum present, since bacteria, freed from the serum in which they had been grown, showed no alteration in virulence. Neufeld (9), in 1902, stated

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that a virulent strain of Pneumococcus, in the course of cultivation, may lose its virulence and its specific agglutinability. Friel (10), in 1915, found that pneumococci, when grown in homologous immune serum, became agglutinable and phagocytable in normal rabbit serum, and less virulent for mice than the untreated strains. Stryker (11), independently, about the same time, reported that the growth of virulent pneumococci in homologous immune serum produced (*a*) variations in agglutinability, (*b*) decrease in virulence, (*c*) inhibition of capsule formation, (*d*) increased phagocytability in normal serum, and (*e*) a change in antigenic properties. She found that the modified type was permanent while the culture was kept on plain media; but that reversion to the original type occurred following animal passage. These investigations preceded the recognition of "R" and "S" forms but the indications are that the avirulent organisms were of the "R" variety. Griffith (2), in the paper in which he first pointed out the morphological distinction between colonies of virulent and attenuated pneumococci, stated that, "an 'R' strain may revert in all respects to the 'S' type or may remain unchanged after many generations in subculture in plain blood broth." He was of the opinion that the constancy of the "R" variant was, in some degree at least, dependent upon the strength of the immune serum employed in its production. None of these investigators made use of single cell cultures, and the contrary results subsequently obtained by Amoss (4) and Reimann (12), were, in part, attributed by them to the fact that they employed pure line strains derived from single cell cultures. Amoss reported that the avirulent strains showed no tendency to revert to the parent type, and did not become virulent on repeated passage through mice. Reimann was unable to restore virulence or type specificity to a single cell "R" strain even after 105 mouse passages. Both Reimann and Amoss employed "R" cultures derived from Type I pneumococcus. Felton and Dougherty (13), by means of an automatic transferring device in a milk-containing medium, were able to restore virulence to a single cell culture of Type I pneumococcus which had become completely avirulent. Although no mention was made of "R" and "S" forms in their communication, it may be assumed that the avirulent cultures were of the "R" variety. They reported, however, that some cultures showed a maximal increase in virulence while others remained avirulent. Levinthal (14) also reported that he was able to effect reversion from "R" to "S" in single cell Type I cultures by growth in serum broth at 25°C., with subsequent passage of the cultures through mice.

The present study concerns itself, generally, with the question of the interconvertibility of "R" and "S" forms of Pneumococcus, and, more particularly, with the question of reversion from "R" to "S." In a previous communication (15), it was pointed out that virulence and type specificity could be restored to "R" forms by growth in anti-"R" sera. Interesting in this connection is the observation of Soule

(16). Investigating microbic dissociation in *B. subtilis*, he stated that, "by the incorporation of 'S' or 'R' immune sera in fluid media, 'R' forms may be obtained from 'S' forms, and 'S' forms from 'R' forms respectively."

Methods.

Both mass and single cell cultures of Types I, II, and III pneumococcus were employed in all experiments. The single cell strains were isolated according to the method of Avery and Leland (17). In this method the position of single organisms is first accurately determined on a thin film of agar spread on a specially designed cover-slip. The single organisms are then allowed to develop into colonies which are subcultured as desired.

The structure and surface appearance of colonies were studied on blood agar plates by means of a Zeiss binocular microscope specially adapted for the study of colony morphology. It is necessary to point out that colony appearance is only a relative guide to the nature of the organisms constituting a colony, and can never be absolutely relied upon to distinguish "R" and "S" forms. This is particularly true in the case of Type II pneumococcus. In this study, colony morphology alone was never considered as a final criterion, but was always confirmed by specific agglutination and virulence tests. The appearance of colonies also shows great variation with age, so that, except in certain instances, plate cultures 18–24 hours old were always selected for examination.

The immune sera employed were either the type-specific, diagnostic sera prepared from horses by the New York State Department of Health,[1] or sera obtained from animals immunized according to the method of Cole and Moore (18).

Unless otherwise indicated, subcultures were made twice in 24 hours, and incubated at 37°C.

EXPERIMENTAL.

I. Reversion from "R" to "S" in Mass Cultures.

A. By Animal Passage.

Mass cultures of "R" forms derived, respectively, from pneumococci of Types I, II, and III, were employed. In each instance these were stock laboratory "R" strains which had been maintained in artificial cultivation at least 2 years, and which showed no tendency to revert to the "S" form by growth in any of the usual media. These

[1] We are indebted to Dr. Augustus B. Wadsworth of the New York State Department of Health for the antipneumococcus horse sera employed in this investigation.

cultures were avirulent, 0.5 cc. of a young broth culture uniformly failing to kill white mice; they did not form capsules or produce the specific soluble substances; and formed only typical rough colonies on blood agar plates.

(*a*) *"R" Forms Derived from Type I (Strain I/192/R).*—It was not found possible to effect reversion of this strain by animal passage. This confirmed the results of Reimann, who had previously subjected the same strain to 105 consecutive mouse passages.

(*b*) *"R" Forms Derived from Type II (Strain D/39/R).*—3 cc. of a 6 hour growth in plain broth were injected into the peritoneum of a mouse and the animal killed in 4 hours. 1 cc. of the peritoneal washings was injected into a second mouse, and the process repeated after 6 hours, the third animal succumbing on the following day. Three further passages were made and a blood broth culture from the sixth mouse proved highly virulent, 0.000001 cc. causing death of an injected animal. The culture now showed only typical "S" colonies, gave a typical Type II agglutination, and produced an abundance of the specific soluble substance. Reversion to the "S" forms had been effected in all respects.

(*c*) *"R" Forms Derived from Type III (Strain M/3/R).*—Much greater difficulty was experienced in causing this strain to revert and it was only after twenty-eight successive mouse passages that it was accomplished. However, reversion did ultimately occur, and it was accompanied by the acquisition of all the characteristics of the "S" form, including maximal virulence.

B. By Growth in Anti-"R" Sera.

The same three "R" strains, derived from Types I, II, and III, respectively, were grown in plain broth, to which 10 per cent of anti-"R" serum obtained from a rabbit immunized to an "R" form was added. In the first experiments the serum employed was from an animal immunized to an "R" form derived from Type III (Strain M/3/R), but it was subsequently found that any anti-"R" serum, provided it was of sufficiently high titer, was equally effective. This phase of the problem will be discussed later.

(*a*) *"R" Forms Derived from Type I (Strain I/192/R).*—No change was effected in this culture even after 100 transfers in 10 per cent

anti-"R" serum. The surface of the colonies became somewhat less rough but there was no increase in virulence and no evidence of return to type specificity.

(*b*) *"R" Forms Derived from Type II (Strain D/39/R).*—This strain, which had remained unchanged after many transfers in blood broth, but which could be reverted to the "S" type by animal passage, showed an unexpected transformation when grown in anti-"R" serum. During four transfers there was no change in the character of the growth, the organisms settling to the bottom of the tube in agglutinated masses, as is usual with "R" forms when grown in anti-"R" sera. On the fifth transfer, however, the media became slightly turbid, and more definitely so on the sixth subculture. On the seventh transfer, the culture showed a uniform turbidity, gave a specific Type II reaction, and proved highly virulent for white mice, 0.000001 cc. causing death. Examination of the colonies on plates showed only typical "S" forms. Evidently complete reversion had been effected.

(*c*) *"R" Forms Derived from Type III (Strain M/3/R).*—"R" forms of Type III pneumococcus reverted to the "S" type, when grown in anti-"R" sera, in a manner similar to "R" forms of Type II. After a variable number of transfers, usually between eight and twelve, the growth became diffuse, smooth colonies appeared on plates, and type specificity, accompanied by maximal virulence, was restored. It is of interest to note that, once the process was initiated, it was only a matter of two or three further transfers before the entire culture had assumed the "S" form, and "R" colonies could not be found.

From these experiments the conclusion may be drawn that, in certain instances at least, mass cultures of avirulent, "R" forms of pneumococci possess the ability to revert to virulent, type-specific, "S" forms, and that the change can be effected by *in vitro* as well as by *in vivo* methods.

Two questions then presented themselves: first, is reversion due to the presence of certain individual, undetected "S" forms within the mass "R" cultures, or do all "R" cells individually possess the ability to revert; and secondly, can reversion be effected by growth in an artificial medium other than that containing anti-"R" serum?

II. Reversion from "R" to "S" in Single Cell Cultures.

Four single cell strains were obtained from each of the three "R" cultures originally derived from the three specific types of Pneumococcus, and their characteristics definitely determined. They were avirulent, 0.5 cc. of broth culture producing no effect on injection into white mice; they showed no evidence of type specificity, and formed only rough colonies on plates. Reversion was then attempted both by animal passage and by growth in anti-"R" sera.

A. By Animal Passage.

The results obtained when single cell cultures were employed so closely parallel those outlined in the case of mass cultures that it is not necessary to give the data in detail. In no instance was it possible by animal passage to effect reversion with the single cell "R" forms derived from Type I (Strain I/192/R). The single cell "R" cultures derived from Types II and III (Strains D/39/R and M/3/R) reverted to the "S" form after practically the same number of mouse passages as was necessary when mass cultures were used. Little or no individual differences were found to exist in the various single cell cultures derived from the same "R" strain.

B. By Growth in Anti-"R" Sera.

The same dilution of serum was used which has been effective in producing reversion in mass cultures. Again entirely similar results were obtained, and practically no individual difference was found to exist in the various single cell strains isolated from the same mass culture. The "R" forms of Type I (Strain I/192/R) uniformly failed to revert; all the "R" forms of Type II (Strain D/39/R) reverted in from five to ten transfers; and the "R" forms of Type III (Strain M/3/R) in from ten to twenty transfers. The "R" forms of Type I were again carried in subculture up to 100 transfers in media containing anti-"R" serum without success.

From the results with single cell cultures, it seems fair to conclude that the reversion of an "R" strain to the "S" type does not depend upon the presence of an admixture of both forms within the culture, but rather it seems not unlikely that each individual "R" organism either may, or may not, possess the ability to revert to the "S" type.

Results with "R" Strains Derived from Other Type I Cultures.

The "R" strain of Type I which was used in the preceding experiments was a stock strain which had been under artificial cultivation for a period of many years. In order to determine whether this inability to revert was a property of all "R" organisms derived from Type I, or only of this particular strain, other "R" cultures derived from Type I were employed. These "R" forms were obtained by growing two Type I "S" strains, recently isolated from cases of lobar pneumonia, in 25 per cent Type I serum. After eight transfers in this medium, four typical "R" colonies, two from each strain, were selected, and reversion was attempted both in mass cultures grown from the single colonies, and in single cell cultures derived from the same colonies. That these cultures were definitely of the "R" forms was repeatedly confirmed by colony appearance, lack of type-specific agglutination, and loss of virulence. In this instance, uniform results were not obtained; but it is of interest to record that the single cell cultures behaved in precisely the same manner as the whole colony cultures from which the single cells were derived. Of the four colony cultures, it was possible, by the method of animal passage, to restore type specificity and virulence to only one. Likewise it was possible to effect reversion by animal passage in single cell cultures derived from this colony and not in the case of the others.

Reversion of these "R" strains of Type I was then attempted in anti-"R" sera. Success was attained with the culture whose virulence could be restored by animal passage but not with the others. Forty transfers in anti-"R" serum broth were necessary and both single cell and mass cultures reverted. Here, again, reversion was accompanied by the acquisition of all the characteristics of the "S" type, including maximal virulence. Thus it appears, at least in so far as Type I is concerned, that there are varying degrees of constancy of the "R" variant. Whether this constancy depends upon the strength of the immune serum employed in the production of the "R" forms as Griffith (2) suggests, or upon the number of transfers in immune serum to which the "R" variant has been exposed, further work is necessary to determine. So far, however, it has always been found possible to effect reversion of "R" forms of Types II and III, single cell as well as mass cultures, both by animal passage and by growth in anti-"R" sera.

Conversion of "S" to "R".

It was thought that possibly some light could be thrown on the difficulty experienced in causing "R" forms of Type I to revert to the "S" type by studying the reverse process ("S" → "R") in greater detail than had previously been done. It was hoped that this procedure would also be of assistance in the interpretation of certain puzzling forms of colonies which were occasionally encountered when "R" forms were grown in anti-"R" sera. "S" strains of Types I, II, and III, were grown through repeated transfers in broth containing the homologous antisera in dilutions varying from 1:1, 1:2, etc., to 1:256.

Type I "S" (Strains I/192/2, "P," and "G").—Each strain went through a more or less similar transformation of which the following account of Strain I/192/2 is typical. Unless otherwise indicated, the description of colonies applies to plate cultures 18–24 hours old.

The growth in 100 per cent Type I serum was poor and plates showed only typical "S" colonies for three transfers. In all serum dilutions, where growth was more abundant, the colonies also remained typically "S" for three transfers before a change was produced. After the fourth transfer, a change became apparent in all dilutions from 1:2 to 1:64—there was great variation in the size of the colonies, without any change in their surface appearance or outline. In higher dilutions only typically "S" colonies occurred. On the fifth transfer, in dilutions 1:2 to 1:64, a small, flat, indistinct, rough colony made its appearance in frequent numbers. As these plates aged (48 hours) a most bizarre picture was produced. Many of the large, smooth colonies developed nibbled areas at the periphery, and the small, flat, rough ones produced a great variety of forms—some developed smooth, nodular papillæ; some a mulberry-like appearance; while others appeared to be undergoing autolysis. After the sixth transfer, the plate presented a still more unusual appearance. Many of the large, smooth colonies showed pronounced nibbling and irregularity of outline, while all the colonies showed a complete lack of uniformity in both size and surface appearance. Occasional "rough" colonies were observed. On the seventh transfer, the majority of the colonies possessed a definitely "rough" surface, but they showed a peculiar "watery" appearance which distinguished them from typical "R" forms. After the eighth transfer, typically "rough" colonies could be recognized but even up

to the fourteenth transfer the small, irregular, rough colony still persisted. Repeated attempts were made to isolate these small, irregular colonies and to establish stable, intermediate forms. These efforts were not successful. Subcultures showed mixtures of the atypical intermediate forms and true "S" colonies.

Type II "S" (Strain D/39/36/2).—In the case of Type II it becomes much more difficult to describe the colony appearance, for the distinction between "R" and "S" forms was much less pronounced and had a purely relative value. In fact it was not unusual to find that a virulent Type II culture would give rise to colonies which appeared rough; while, on the other hand, colonies which were apparently smooth, especially when grown in blood or serum broth, were not necessarily type-specific. However, it is possible to say that the transformation from Type II "S" to the "R" form occurred much more readily than in Type I, one exposure to type-specific serum often proving sufficient. Moreover, the unusual transition picture described in Type I was not encountered, possibly because of the readiness with which the transition to the "R" form occurred.

Type III "S" (Strain A66/47/2).—With Type III it was always possible to distinguish "R" and "S" colonies. After one transfer in homologous anti-"S" serum, in all dilutions, only "S" colonies occurred. After two transfers only a few "S" colonies persisted in serum dilutions 1:2 and 1:4; in higher dilutions only "S" forms were apparent. After four transfers, dilutions 1:2 and 1:4 showed only "R" forms; in dilution 1:8 only two "S" colonies remained. After the sixth transfer, all the colonies were of the "R" type in dilutions 1:2, 1:4, and 1:8, while in higher dilutions, only "S" forms persisted. No intermediate forms were observed, all colonies being definitely either "R" or "S."

From these experiments it is possible to state that Type I "S" pneumococcus is by far the most difficult to convert to the "R" form, requiring ten to fifteen transfers in serum dilutions of 1:2 and 1:4. Even after this number of transfers "intermediate" colonies exist which readily revert to the "S" type. On the other hand, Types II "S" and III "S" are easily converted to the "R" form, and in each instance the change is much more abrupt and complete than in the case of Type I. This fact may offer a partial explanation of the difficulty experienced in the reverse process of transforming "R" forms derived from Type I to the "S" type.

The Rôle of Anti-"R" Serum in Reversion by in Vitro Methods.

Having established the fact that, in the majority of instances, it is possible to transform "R" cultures of Pneumococcus to the "S" type by growth in anti-"R" sera, attempts were made to effect the change in media which did not contain anti-"R" antibodies. Experiments were also carried out to determine the titer and concentraton of serum best suited for the purpose.

Sera were obtained from a series of normal individuals and animals, and also from animals which had been immunized to either the "R" or "S" forms of Pneumococcus. Attention is drawn to the observation of Avery and Heidelberger (19) that type-specific, antipneumococcus

TABLE I.

Reversion "R" to "S"—Type II Pneumococcus. Growth in Normal and Anti-"R" Sera.

Culture	Serum broth dilutions 10 per cent	Anti-"R" titer of sera	No. transfers	Result
"R" forms of Type II (D/39/R). Single cell culture	Normal chicken	Nil	100	No change
	" rabbit	1:5	100	" "
	" sheep	1:20	100	" "
	" horse	1:20	100	" "
	" guinea pig	1:320	30	Partial reversion
	" human	1:640	8	Complete "
	Immune rabbit	1:3200	5	" "
	" horse	1:3200	5	" "
	Plain broth		100	No change

sera contain not only dominant, type-specific (anti-"S") antibodies, but also antibodies reacting with the protein substance, which is common to all pneumococci. Reimann (12) subsequently showed that sera prepared with "R" forms are immunologically similar to sera prepared with the protein of Pneumococcus. In consequence it was possible to use not only anti-"R" sera prepared against any "R" strain regardless of its type derivation, but also anti-"S" sera of the three specific types, all of which possess, in common, anti-"R" antibodies. The anti-"R" titer of the sera was determined by the so called thread reaction, and single cell cultures were employed.

The results obtained with "R" forms of Type II pneumococcus are set forth in Table I. It is seen that complete reversion occurred only in those sera of which the anti-"R" titer was above 1:320. In normal guinea pig serum, the titer of which was 1:320, a partial reversion was observed. Under these conditions the organisms lost their ability to thread; the colonies became smoother; and the virulence of the culture was increased, but not to a maximal degree. It was not probable in this instance that the growth was made up of a mixture of "R" and "S" forms; for all the colonies were of similar appearance and sub-

TABLE II.

Reversion "R" to "S"—Type III Pneumococcus. Growth in Normal and Anti-"R" Sera.

Culture	Serum broth dilutions 10 per cent	Anti-"R" titer of sera	No. transfers	Result
"R" forms of Type III (M/3/R). Single cell culture	Normal chicken	Nil	50	No change
	" goat	"	50	" "
	" horse	1:10	50	" "
	" sheep	1:20	50	" "
	" rabbit	1:20	25	Complete reversion
	" guinea pig	1:80	50	Partial " ?
	Immune goat	1:200	50	No change
	Normal human	1:640	50	" "
	Immune sheep	1:800	50	" "
	" rabbit	1:3200	18	Complete reversion
	" horse	1:6400	12	" "
	Plain broth		50	No change

cultures made from them individually possessed the same characteristics. Even when subcultured for twenty further transfers in the same media complete reversion did not occur. Growth in serum broth with an anti-"R" titer of less than 1:320 induced little change. In some instances the organisms ceased to give a thread reaction, but there was slight, if any, increase in virulence. They did not give a type-specific agglutination and were still unquestionably "R" forms.

The corresponding results obtained with "R" forms of Type III are outlined in Table II. Reversion again occurred promptly in those sera the anti-"R"content of which was highest, and not at all in those

which did not contain anti-"R" antibodies. A partial exception occurred in the case of normal rabbit serum, the anti-"R" titer of which was only 1:20. Here complete reversion was effected after twenty-five transfers. This was the only instance encountered in which the transformation took place in sera of low anti-"R" titer. This observation suggests that, while reversion unquestionably occurs much more readily in sera of high anti-"R" content, it may take place occasionally even in sera of low anti-"R" titer.

The results obtained by growing "R" forms of Type III in normal guinea pig sera are of some interest. A great variety of colony appearance was produced which closely resembled that previously described

TABLE III.

Optimal Concentration of Anti-"R" Serum for Reversion.

Culture	Serum concentration	No. transfers to effect reversion
"R" forms of Type II (Strain D/39/R)	25 per cent	8
	10 " "	4
	5 " "	10
	1 " "	No change
"R" forms of Type III (Strain M/3/R)	25 " "	16
	10 " "	7
	5 " "	20
	1 " "	No change

in the degradation of Type I "S" cultures. After fifteen transfers in this medium, colonies quite irregular in size, outline, and surface appearance were observed. In addition to the more usual "rough" forms there were small, indistinct, flat, smooth colonies; small, rough ones from which projected smooth nodular papillæ; and medium sized, apparently smooth colonies. All of these types of colonies appeared on plate cultures after 18 hours incubation. After ten further transfers only smooth colonies occurred but they were not of the large, mucoid, typical, Type III variety and subcultures from them were avirulent and not type-specific. Continued transfers in normal guinea pig serum produced no change and when transferred back to plain broth only typical "R" forms resulted. Possibly this culture represented an unstable intermediate form.

Optimal Concentration of Anti-"R" Sera for Reversion.

Cultures of "R" forms of Types II and III pneumococcus were grown for successive transfers in broth containing varying dilutions of anti-"R" rabbit serum of known titer. The results are recorded in Table III. The anti-"R" titer of the serum used, as determined by the thread reaction, was 1:6400. In all experiments 10 per cent was found to be the optimal concentration. Reversion also occurred in serum dilutions of 25 per cent and 5 per cent but a greater number of transfers was necessary.

Experiments were also done with sera which had been inactivated by heating at 56° for ½ hour, and it was found that this procedure did not reduce the anti-"R" titer nor destroy its ability to cause reversion. No work was done under anaerobic conditions nor were the sera of animals immunized to the nucleoprotein of Pneumococcus tested.

DISCUSSION.

The present work establishes the fact, that, in the majority of instances, it is possible to cause both mass and single cell "R" cultures of Pneumococcus to revert to the "S" type. It was found that there are differences in the stability of the "R" forms in the three types, "R" forms of Type I being the most permanent. This corresponds to the greater resistance to degradation exhibited by Type I "S." Absolutely irreversible "R" organisms were encountered only in Type I. No attempt was made to determine whether it is possible to produce such irreversible forms in Types II and III.

It is possible to cause reversion from "R" to "S" pneumococci by an *in vitro* as well as an *in vivo* method. Reversion from "R" to "S," whether effected *in vitro* or *in vivo*, is always accompanied by the acquisition of maximal virulence. The *in vitro* method consists in growing "R" organisms in anti-"R" sera, the optimal concentration of which is 10 per cent. Growth in sera which do not contain anti-"R" antibodies, has, with one partial exception, not been found to be effective in producing the transformation. The partial exception was that of a normal rabbit serum, the anti-"R" titer of which, as determined by the thread reaction, was 1:20. "R" cultures have invariably reverted to the specific type of Pneumococcus from which the

"R" organisms were originally derived. After reversion, the culture possesses maximal virulence, elaborates the specific soluble substance, and is type-specific. No explanation is offered for the causes responsible for the change.

In our experience single cell strains have always reacted in the same manner as the mass cultures from which they were isolated. This finding argues against the hypothesis that the virulence of a culture depends upon the relative number of "R" and "S" forms of which it is composed.

The observation that normal human sera contain sufficient anti-"R" antibodies to cause reversion of some "R" cultures is thought to be of some interest.

It is not the purpose of this paper to enter into a discussion of the epidemiological significance of these findings, nor the bearing they may have on the subject of bacterial mutation.

CONCLUSIONS.

1. Single cell cultures of "R" pneumococci may revert in all respects to the "S" type.

2. In all instances in which reversion has occurred the "R" forms have invariably reverted to the same specific type from which they were originally derived.

3. The transformation "R" to "S" may be effected *in vitro* by growth in anti-"R" sera, as well as *in vivo* by the method of animal passage.

4. Reversion from "R" to "S," whether effected *in vivo* or *in vitro*, is always accompanied by the acquisition of maximal virulence.

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