PNEUMOCOCCUS VARIANTS

I. INTERMEDIATE FORMS AND THE INFLUENCE OF ENVIRONMENT IN THEIR PRODUCTION DURING IN-VITRO S TO R AND R TO S TRANSITIONS

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Received for publication October 31, 1933

One method of studying the problem of variation in the pneumococcus consists of an enumeration or a classification of the various bacterial "forms" which arise progressively during the process in which S pneumococci lose their ability to elaborate soluble specific substances and R pneumococci gain this ability, or in other words during the degradation of S forms, or the reversion of R forms. This amounts essentially to a series of lines which are drawn more or less arbitrarily through certain involuntary or evolutionary stages of the dissociative process. Such an approach is justified, however, by the fact that Blake and Trask (1923 and 1928) have shown that several, fairly stable pneumococcus variants intermediate between S and R, can be produced with a considerable degree of consistency, and that they are recognizable by characteristic properties. Upon this fact rests the basis of the analysis employed in this communication.

TAXONOMIC LITERATURE DEALING WITH INTERMEDIATE AND R PNEUMOCOCCI[1]

The effort will not be made in the present communication to review literature which deals with the development of knowledge

[1] The use of the term R pneumococcus in this paper (as well as the term S) is in accordance with the terminology generally employed during the past decade. Recently Dawson (1933b) has suggested that still further revision of terminology may be necessary, with the term M (mucoid) supplanting the term S; the term S supplanting the term R; and the term R being applied to a new variant which he has described (1933a).

concerning pneumococcus variants. Articles covering this field to which the reader may be referred include those of Kimura, Sukneff and Meyer (1928); Neufeld and Schnitzer (1928); Gundel (1931), and Blake and Trask (1933). It may suffice to say, however, that apart from the recognition of the so-called fixed types of S pneumococci other forms have long been described, which at first were spoken of as "atypical pneumococci" or "streptococci." Later the term rough or R pneumococcus was introduced by Griffith (1923) to cover a large group of variants which have been derived from S types I, II, and III and also from group IV. The term R pneumococcus has been used somewhat loosely perhaps, to designate those variants which produce small, rough-surfaced colonies in contrast to the larger, smooth colonies of the S forms. R pneumococci have also been broadly characterized by avirulence for mice, and loss of the specific polysaccharide to which the "parent" S strains owe their type specificity. They generally retain the ability to be agglutinated by the species-specific fraction of anti-pneumococcus serum, and according to most observers are soluble in bile, although this latter property may be somewhat impaired. That there may be various degrees of stability or "roughness" of the R forms, as expressed in terms of the ease or difficulty with which either spontaneous or induced reversion takes place, has been intimated by several, and this question will be elaborated in the following paper.

The existence of forms intermediate between S and R has also been described. The main features differentiating the intermediate group are that they produce colonies of which the surface is either smooth, granular, or partially but not wholly rough, and that different forms show characteristic degrees of virulence and agglutination patterns. A classification of this intermediate group, first outlined by Blake and Trask (1923), has been recently published by these authors (1933). As their scheme of classification represents the basis of methods followed in this paper, it will be given in some detail. The intermediate variants described by Blake and Trask were produced by growing S forms (types I and II) in homologous anti-pneumococcus serum broth,

and were identified by their colony appearance on rabbit-blood agar plates; their agglutination reactions in homologous and heterologous anti-pneumococcus serum according to the thread test technique, and their virulence for mice. Five variants intermediate between S and R were described which appeared progressively during the degrading process to which a strain of I-S pneumococcus had been subjected. The same general dissociative response was also observed by these authors with II-S strains.

Our own experiences with the application of Blake and Trask's classification and the characteristics whereby we have identified the intermediate variants (designated as I-*a* to I-*e*, inclusive) appear in table 1. Similar characteristics of a I-R strain have also been listed in this table. It will be seen that the major feature in the differentiation of these intermediates has been that of the thread test agglutination reaction based upon various ranges in titre of anti-S agglutination, demonstrable as a firm disc or large flakes; and of anti-P (or anti-R) agglutination, which is quite different and consists of a fine suspension of tiny particles. It should be noted in particular, however, that cross agglutination, *i.e.*, agglutination in low dilutions of heterologous sera first begins to appear in the I-*b* variant; and that complete loss of the coarse flaking (anti-S agglutination) first appears in the I-*c* variant. For the application of Blake and Trask's classification it should also be emphasized that it was devised from stabilized strains, and that the original agglutination reactions were based upon one particular lot of type I anti-pneumococcus serum. Freshly isolated intermediate strains have been often found which present over-lapping properties, and furthermore different lots of anti-pneumococcus serum show a different anti-S and anti-P content, but by and large the classification has proved to be one which may be readily applied and has been used in this laboratory for the past ten years.

A slightly different terminology was subsequently used in Germany by Neufeld and Levinthal (1928), who employed the term -s to designate certain forms intermediate between S and R which were of low virulence for mice or completely avirulent,

TABLE 1

Blake and Trask's classification of pneumococci intermediate between I-S and I-R

PNEUMOCOCCUS	SERUM TYPE	AGGLUTINATION REACTIONS (THREAD TEST) 1:20	1:40	1:80	1:160	1:320	1:640	COLONY Surface and size	Hemolysis	RELATIVE STABILITY OF STRAIN	MOUSE INOCULATION M.L.D.	Days for M.L.D. to kill	Organism generally recovered
I-S	I	+++	+•+	−	−	−	−	Smooth and large	No	Quite stable	10^{-7} to 10^{-8}	1–2	I-S
		+•+	+•+	+	+	+	±						
	II	−	−	−	−	−	−						
I-*a*	I	+	+•+	+•+	++	+	−	Smooth slightly smaller than I-S	No	Unstable tends to revert to I-S			
		+	+•+	+•+	++	++	+						
	II	−	−	−	−	−	−						
I-*b*	I	+	+	+•+	+•+	++	+	Smooth slightly smaller than I-*a*	No	Quite stable	10^{-2} to 10^{-4}	2–4	I-*b*
		±	+	+	±	±	±						
	II	±	−	−	−	−	−						
		+	+	±	−	−	−						
I-*c*	I	±	±	±	±	±	+	Smooth about ½ size I-S	Slight hemolysis may be present	Quite stable	0.5 cc. to 10^{-2}	3–7	I-S
		++	++	+	+	±	±						
	II	+	±	±	±	−	−						
		++	++	+	+	±	±						

I-*d*	I	±	±	±	±	±	±	Smooth or slightly granular, very small	Wide zone of hemolysis	Unstable, dissociates to I-*c* and I-*e*	0.5 rarely kills	3	I-S
	II	±	±	±	±	±	±						
I-*e*	I	±	±	±	±	±	±	Smooth or slightly granular, ½ size I-S	Narrow zone of hemolysis	Unstable, dissociates to I-*c*, I-*d* and I-R			
	II	±	±	±	±	±	±						
I-R	I	++ +	++ +	+ −	± −	± −	± −	Rough or granular	Slight hemolysis may be present	Quite stable	0.5 does not kill		
	II	++ +	++ ±	+ −	± −	± −	± −						

Legends:

+++ = firm disk

+•+ = disk easily broken

+ = few large flakes

++ = heavy agglutination

+ = fine agglutination

± = very fine agglutination

− = no agglutination

and which gave rise to smooth-surfaced dome-shaped colonies generally smaller than those produced by S forms. Using this terminology as a basis, Klumpen (1932) has recently made an analysis of the intermediates. This author relied essentially upon colony form for his classification. His dissociative patterns were induced by growing S forms in various concentrations of homologous anti-pneumococcus serum, and in media containing animal tissues according to the method of Neufeld and Levinthal (1928). Klumpen used the following terms to designate three intermediate colony types which appeared in the order named: -s colonies, which are described above; -U colonies (Ubergängsformen) characterized by disc-shaped colonies of about the same size as that of an -s with smooth or rough surfaces, in some of which the border was smooth and the center rough; and RK or giant colonies (Riesenkolonien). The latter were very large and frequently had smooth surfaces. They appeared shortly before the true Rs were noted. Other properties of the organisms giving rise to these three types of colonies are described by Klumpen as being unstable. Some were type-specific and virulent, others were not.

Klumpen's -s colonies probably correspond to those produced by Blake and Trask's *-a* and *-b* forms; his -U colonies probably correspond to those of the *-c* forms. We have not had occasion to observe his RK or giant colonies.

GENERAL METHODS

In this communication the terminology employed by Blake and Trask will be followed. The use of the term R pneumococci is restricted to those avirulent forms which give a characteristic R type of thread-test agglutination (see table 1), and produce colonies which have a rough surface, similar to those shown in figures 7 to 9.

Agglutination reactions

These were done in all instances by the so-called thread-test method, in which organisms were grown in a series of six dilutions of homologous and heterologous anti-pneumococcus sera. The method is well known, but as it represents the major basis of our differentiation of the variant groups, a detailed description of the technique employed for testing variants derived from I-S, II-S and III-S strains will be given.

Primarily a series of stock dilutions of anti-pneumococcus sera (types I, II, III)[2] are made up in meat, or yeast extract broth (pH 7.2) in concentrations representing 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640. These are stored in large test tubes and kept in the ice-box. It is of primary importance to use a broth diluent in which fresh strains of S pneumococci will grow without the addition of growth enhancement substances such as blood cells, serum or glucose. We have lately employed for this purpose a modification of a yeast extract broth recommended by Rakieten (1932), which will presently be described.

For each individual test thirteen sterile agglutination tubes (inside diameter 9 to 10 mms.) are employed. The tubes are set up in two rows, a front row of seven, and a back row of six. To the first six tubes of the front row 0.5 cc. of dilutions (1:20 to 1:640) of type I anti-pneumococcus serum are introduced, an equal amount of plain broth is placed in the seventh or control tube. To the back row of 6 tubes similar dilutions of type II anti-pneumococcus serum are introduced. If the organism to be tested is known to be derived from type III, this type of serum dilutions is substituted in the back row. The tubes are then inoculated with one drop from a capillary pipette of a sixteen-hour blood-broth culture of the strain to be tested. They are incubated over night and read the following morning. As is well known the interpretation of this test requires careful controls, for the character of agglutination obtained is dependent upon at least three known factors: (a) the degree of growth, which should neither be too sparse nor too heavy; (b) the relative potency of the particular lot of anti-pneumococcus serum, as different lots seem to differ in their anti-S and anti-P content; and (c) acid agglutination phenomena. The last factor may vary somewhat in different lots of broth depending upon buffer substances and acid producing substances such as glucose. To control the former we have used broth which contains a high concentration of sodium phosphate. The formula for the type of broth which in our hands has during the past year proved satisfactory for use in the thread test may be given as follows:

10 grams yeast extract (Savita)
10 grams "Bacto" peptone[3]
2.5 grams sodium chloride

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[2] I am indebted to the Division of Laboratories, New York State Department of Health, for the anti-pneumococcus sera employed in these experiments.

[3] Since this article was written we have substituted Neopeptone for "Bacto" peptone.

8 grams sodium phosphate ($Na_2HPO_4 \cdot 12\ H_2O$)
1000 cc. H_2O

The Savita, "Bacto" peptone and sodium chloride are dissolved in a liter of water. The reaction is set at pH 7.5 and the sodium phosphate is then added. The medium is autoclaved for fifteen minutes at 15 pounds pressure. It is then filtered through filter paper, bottled in 50 cc. lots and again autoclaved for ten minutes at 10 pounds pressure.

Before any new lot of broth is employed tests should be made as to whether it will yield a good growth when inoculated with virulent and preferably freshly-isolated pneumococci. Subsequently a series of control tests should be set up with organisms of known and characteristic agglutination reactions such as I-S, I-*b*, and I-R.

Colony studies

Individual colonies, representing 36 to 48 hour growths on fresh rabbit-blood agar plates were examined under the low (16 mm.) power objective of the microscope by reflected light from the tilted surface of the plate. As the appearance of pneumococcus colonies is in some measure dependent upon the medium upon which they are grown, it is important that stable control strains of S and R pneumococci should be frequently grown on the same plate for comparison with colonies of the strains to be investigated. In general, plates were streaked to give rise to as many discrete colonies as possible. Particular attention was paid to the surface i.e., smooth, granular, or rough; the size; the configuration, i.e., dome, disk or crater shaped; the outline, i.e., round or irregular; the associated peripheral zone of "greening" of the medium; or a zone of hemolysis; the degree of autolysis, and the development of daughter colonies. In determining colony size at least ten of the largest discrete colonies were selected and their diameter measured by inserting a micrometer eye-piece into the microscope. Several sample colonies representive of those usually encountered in S, intermediate, and R forms appear in figures 1 to 9. In these figures the attempt has been made to show typical and atypical colony appearances, as, for instance, those exhibited by smooth colonies. The surface of the forty-eight-hour S, -*a*, and -*b* colonies is smooth, but when (after twenty-four to seventy-two hours) autolysis has taken place, which is a major characteristic of smooth pneumococcus colonies, a faint pseudo-roughening of the surface may occur (see figs. 2 and 3).

Mouse virulence

The terms *virulence* and *avirulence* have been employed somewhat loosely in the recorded results to designate whether or not 0.5 cc. of an 8- to 16-hour blood broth culture injected intraperitoneally, would kill a mouse. An organism was considered to be avirulent when 0.5 cc. of culture failed to kill within 10 days.

When virulence titrations were done, broth dilutions ranging from 10^{-1} to 10^{-8} were made from an eight-hour glucose blood broth culture, and five mice were inoculated with 0.5 cc. from the five highest dilutions. Plates were poured for colony counts with 0.5 cc. portions of the dilutions higher than 10^{-5}. Unless there were approximately eight or twelve colonies in the plate poured from the 10^{-7} dilution, the result was considered unsatisfactory.

Dissociation of S strains in homologous anti-sera

In our hands this method has proved the easiest and most reliable way of readily producing intermediate and R pneumococci. Representative I-S and II-S strains were grown in broth containing 10 per cent homologous anti-sera. The cultures were transferred daily and at appropriate intervals subcultures were made upon blood agar plates. The colony, the mouse virulence, and the thread-test agglutination of strains picked from single colonies were studied.

Reversion experiments

These were performed by the *in vitro* method described by Dawson (1928), which consists of carrying daily transfers in 10 per cent serum broth using either plain rabbit serum or anti-R serum prepared by the subcutaneous and intravenous inoculation of rabbits with strains of R pneumococci. We are not prepared to say whether anti-R serum broth is actually preferable to plain rabbit serum broth for this purpose. During the first two years in which these experiments were carried out anti-R serum broth was used, and during the latter two years plain rabbit serum broth has been used. Results with the latter method seemed to be as satisfactory as with the former.

Individual forms picked from single plated colonies were studied during the reversion process in the same manner as in the degradation process.

Strains employed

A number of strains of pneumococci were employed in the course of these experiments. They will not be described individually. It may suffice to say that our effort was to test freshly isolated as well as stock S strains. Thus several I-S and II-S strains recently isolated from the blood or sputa of patients suffering from pneumonia or suppurative pneumococcus infections were included; also a highly virulent, stabilized II-S strain which had been subjected to frequent mouse passages over a period of many years;[4] and a number of S, *-a*, and *-c* strains which had been kept on blood agar plates for several months or years. Two stock R strains, I-R and II-R were also constantly used as control strains.[5] Obtained in 1926 they have been kept on blood agar plates with transfers every three or four weeks, and will be described more fully in the following paper.

EXPERIMENTAL

Dissociative patterns in S → R transitions

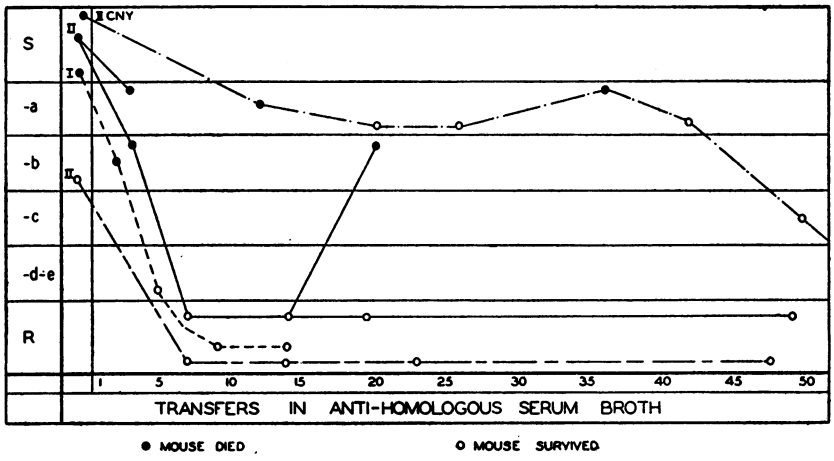
In order to note the consistency with which intermediates arose in the course of S → R transitions with different S strains, a series of experiments was first run in which daily transfers were made in 10 per cent homologous anti-serum broth. The dissociative response induced in a I-S, two II-S, and a I-*b* form is shown diagrammatically in text-figure 1. Individual variants listed in this figure merely represent the predominant form or forms observed on plated cultures in which agglutination tests and mouse inoculations were done. No attempt has been made to show the relative percentage of two forms when they appeared together. Furthermore, as mentioned above, no attempt has been made to stabilize these individual variants, but merely to classify them by their thread test reactions, virulence and colony type, *as they arose in the course of the dissociative process.* This feature may be in part responsible for the discrepancies which seem to exist between the virulence and the agglutination reaction of some of the *-a* to *-e* forms listed in these experiments

[4] I am indebted to Miss Georgia Cooper of the Bureau of Laboratories, Department of Health, City of New York, for this strain.

[5] I am indebted to Dr. O. T. Avery of The Rockefeller Institute for Medical Research for these strains.

when compared with the stabilized -*a* to -*e* strains listed in table 1.

The dissociative responses recorded in text-figure 1 are essentially in accord with those noted by others, namely, the variants successively produced generally follow a fairly rapid progression during their transition to R forms. It will also be noted that there are differences in the rate of dissociative response in some of the experiments. Such differences may be due to unknown variables for it has been our experience that it is difficult to pre-



TEXT-FIG. 1. Dissociative patterns induced in three S strains, and a II-*b* strain by growth in homologous anti-pneumococcus serum broth. The major trend is that of rapid "degradation" with the production first of intermediate forms and then stabilization of the strain as an R. The response of one of these strains (II CNY) was, however, much slower than that of the others.

dict, except within wide limits, just what the dissociative response of a given strain will be, even though the identical experiment be repeated several times. On a few occasions not recorded in text-figure 1, we have even noted the rapid transition of S to R forms on the second or third transfers without having previously detected intermediates. There seem to be some measurable variables, however, which influence the dissociative rates induced by this method. These include: (a) differences in the potency of the particular lots of anti-pneumococcus serum employed; and (b) differences in the susceptibility or stability of the individual

strains. For instance the II-S (CNY) strain is an example of the latter effect. It had previously been subjected to mouse passages for a long period (see under Methods) and responded more slowly to the anti-serum. In this strain, although there was a transient period of loss of virulence between the twentieth and thirtieth transfers, virulence was subsequently regained and was present on the thirty-seventh transfer. The fiftieth transfer revealed only avirulent II-*c* forms.

It further appears in these and other similar experiments that the end stage in the degradation process induced by this method, was essentially that of the R form. In a single instance, not shown in text-figure 1, we have carried a strain in which R forms appeared on the twelfth transfer, to the eighty-fifth transfer and it still yielded only typical R forms with an occasional spontaneous throw-back to a virulent intermediate form such as that recorded in the twentieth transfer of strain II-S.

Dissociation of S Strains in Media Containing Bile

It is recognized that if S pneumococci are grown in successive transfers of broth originally containing small but increasing amounts of bile, dissociation to R forms may be induced. This procedure has been used by Reimann (1925), who employed the method of rapidly transferring S cultures in broth containing a 1:400 dilution of bile, later 1:200, and finally after 69 transfers, growth was maintained in broth containing 75 per cent of bile.

Methods of growing S pneumococci in bile

In our attempts to bring about variant production through growth in bile, difficulty was frequently encountered in maintaining the viability of cultures which were successively transferred, so we resorted to growing pneumococci in a continuous flow of broth to which bile was gradually added.

The continuous culture apparatus was a modification of that described by Felton and Dougherty (1924), in which the major alteration was that the rate of flow of medium was adjusted by a screw clamp instead of an automatic electrical device. The apparatus was adapted for use in an ordinary incubator in which holes were bored in the top and sides to allow for entrance and exit tubes. Meat extract broth (pH 7.2) was

employed, to which measured amounts of ox bile (which had been sterilized in the autoclave) were added. The culture was maintained in a U-tube of 100 cc. capacity. At the beginning of each experiment the apparatus was filled with broth from a 2-liter graduated reservoir bottle and the U-tube was then inoculated with 1 cc. of a fresh blood broth culture of the strain to be tested, together with a few drops of fresh rabbit blood for the initiation of growth. The rate of flow was then adjusted so that initially between 75 to 100 cc. of media passed through the apparatus every twenty-four hours. Measured amounts of bile were then slowly added to the reservoir bottle from a graduated cylinder of 500 cc. capacity. Daily readings of the rate of flow through the apparatus were recorded together with a record of the increasing per cent of bile in the reservoir. Cultures, taken from a sampling tube, were made at intervals of a few days and the characteristics of the organisms thus isolated were studied.

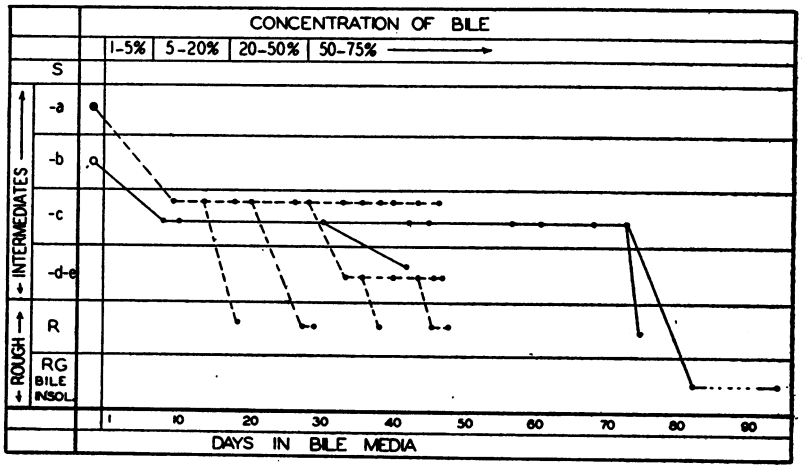
It was found that a culture of S pneumococci inoculated into a continuous flow of medium could survive the rapid addition of far higher concentrations of bile than we had been able to use in our preliminary experiments of transferring the cultures from tube to tube. Provided a rapid rate of flow was at first maintained, this concentration could be increased to from 1 to 5 per cent within the course of a few days and subsequently to much higher concentrations. Eventually the flow could be diminished or stopped for many days at a time. Another feature which should be mentioned is that when medium containing bile reached the U-tube of the continuous culture apparatus the usual evidences of bacterial growth disappeared, and the medium became quite clear with a brownish sediment at the bottom of the tube. However, samples of this relatively clear fluid yielded growth of pneumococci on blood plates. The period in which cultures of pneumococci would remain viable in this apparatus was largely dependent upon the rate at which fresh medium was added. Our usual experiment lasted from fifty to one hundred days.

Occasionally towards the end of an experiment, when very few colonies appeared on the plates inoculated from the sampling tube, cultures on the next one or two days would yield no growth, but on the subsequent day or days, a few colonies would again be found. When cultures remained sterile for more than a week the experiment was terminated.

Dissociative patterns obtained in bile

The object of these experiments was to compare the dissociation of S strains with that induced by growth in anti-pneumo-

coccus serum broth. Six experiments were carried out in which cultures were maintained in a continuous flow of medium containing bile for from fifty to one hundred days. The results of two of these experiments appear in text-figure 2. The dissociative patterns will not be described in detail for in general the "degradation" process induced, resembled that brought about by antipneumococcus serum, but the S → R transition seemed to take place more slowly, and the major trend was for the organism to become more or less stabilized as intermediate *-c* or *-d* forms,



TEXT-FIG. 2. Dissociative patterns induced in two strains (I-*a* and II-*b*) in media containing bile. The I-a dissociants are shown as small dots, the II-b dissociants as small circles. The major trend is that of stabilization as *-c* and *-d* forms with the occasional production of R forms, and rarely bile insoluble so-called RG forms.

rather than as R forms. Many R forms were, however, produced generally after twenty or thirty days; most of them were partially bile insoluble, a few bile insoluble. Furthermore, in the latter part of one experiment (see text-figure 2) a bile insoluble strain was isolated which produced very rough colonies and a granular growth in broth. This type of variant, which will be discussed in the following publication, has been designated as an RG (bile insoluble form). It was only observed on two occasions at the termination of one experiment, the other five experiments were terminated by the death of the dissociating strain.

TABLE 2

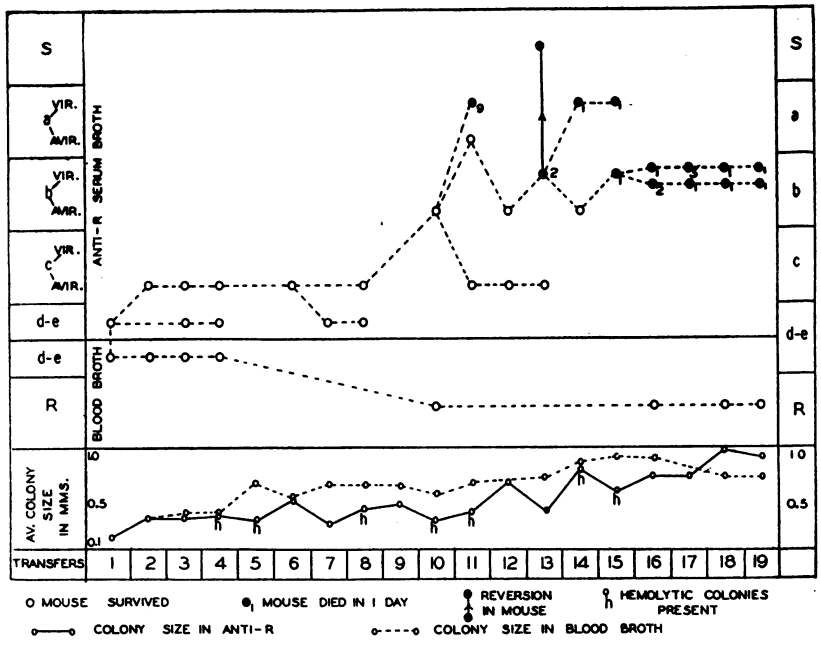
Agglutination reactions and other characteristics of forms arising during in vitro reversion of I-R → I-b

TRANSFER	ORGANISM FROM ANTI-R SERUM BROTH							MOUSE INOCULATION	ORGANISM FROM MOUSE					
	Colony surface	Serum type	Agglutination reactions 1:20	1:40	1:80	1:160	1:320	Form		Agglutination reactions 1:20	1:40	1:80	1:160	1:320	Form
1	Rough	I	+	+	±	±	±	I-R	Survived						
		II	±	±	±	−	−								
4	Granular (smooth?)	I	+	+	+	+	±	I-*c*?							
		II	±	+	+	±	±								
8	Smooth	I	+	+	±	±	−	I-*c*	Survived						
		II	±	±	±	−	−								
12	Smooth (hemolytic)	I	+	+	+	+	±	I-*c*							
		II	±	±	±	±	−								
16	Smooth	I	+	+	+	+	±	I-*c*	Survived						
		II	+	+	+	±	±								
20	Smooth	I	+	+	+	+•+	+•	I-*b*	Died 4 days	+	+	+•+	+•+	+•+	I-*b*
		II	−	±	±	±	−			−	−	−	±	−	
24	Smooth	I	+	+	+	+•+	+•+	I-*b*	Died 3 days	+	++	+•+	+•+	+•	I-*a*
		II	±	+	+	+	±			−	−	−	−	−	

Legends as in table 1.

In vitro reversion patterns of R and intermediate forms

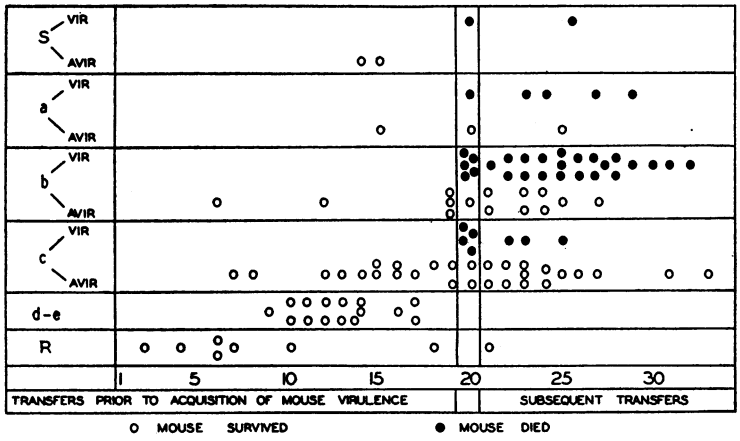
In the reversion process induced by the growth of -*c*, -*d*, or R forms in 10 per cent anti-R serum broth or in plain rabbit serum broth, a fairly consistent pattern also resulted. Some R strains proved refractory and failed to show any change after one hundred or more transfers in this environment. When re-



TEXT-FIG. 3. Dissociative patterns of a II-*d* strain in two different types of media. In anti-R serum broth reversion occurs in which the eventual trend is that of stabilization as a II-*b*; in blood broth the strain is stabilized as a II-R. The gradual increase in the average colony size in both of these environments is shown by graphs in the lower portion of the figure.

version was induced, however, intermediate variants were regularly produced in the reverse order from that in which they appear in the S → R transitions. All of the strains reverted by this method went back to the same type from which they had been originally derived. A single experiment in which reversion of a I-R to a I-*b* was induced by growth in anti-R serum broth, is shown in table 2. A single colony, representative of the pre-

dominant form on the plated cultures from individual serum broth transfers, was picked daily and from this culture a mouse was inoculated and the agglutination reaction was determined. It will be seen that by the eighth transfer the colonies had become definitely smooth I-*c* forms, maintaining this form until the twentieth transfer when a I-*b* type of agglutination was first noted and the organism had also acquired the ability to kill a mouse in four days. On the twenty-fourth transfer reversion from I-*b* to I-*a* took place as a result of the mouse passage.



TEXT-FIG. 4. Composite reversion patterns in a series of eleven strains originally representing -R, -*d*, and -*c* forms, induced by growth in anti-R serum broth and in plain rabbit serum broth.

A similar experiment is shown in text-figure 3. Here the original strain, a II-*d*, was carried for twenty transfers in anti-R serum broth and also simultaneously in blood broth. Two colonies were picked each day for study. The diagram in text-figure 3 illustrates the apparent complexity of the dissociative pattern in which this labile strain became stabilized in two different environments, in one of which partial reversion took place, and in the other slight degradation. With the former, or in anti-R serum broth, the colonies gradually became larger and smoother and by the eleventh transfer organisms from one colony, which gave a II-*a* type of agglutination, had acquired the ability to kill a

mouse in nine days. II-*a* forms were again found on the fourteenth and fifteenth transfers, but eventually the culture became more or less stabilized as a II-*b* maintaining this form from the fifteenth to the nineteenth transfer. The virulence of a culture picked from a colony on the nineteenth transfer was found to be 10^{-4}

It is of some importance to note that the only II-*b* form which reverted to a higher form in a mouse was among the first of the II-*b* forms to appear. This recalls a feature which has been noted by Blake and Trask (1933), namely, that when forms of low virulence (particularly -*c* forms) kill mice, it generally takes three to seven days for the mouse to die but the organism recovered from the dead mouse is usually found to be an S or an -*a* of high virulence. On the other hand, -*b* variants kill mice more readily and more quickly but do not show as much tendency to revert to a higher form during the process.

Conversely, in the blood broth transfers the original II-*d* strain gave colonies which also gradually increased in size, but became rougher instead of smoother. By the tenth transfer only R forms were found. This stabilized R strain subsequently failed to revert when carried for twenty transfers in the same anti-R broth in which reversion had been induced in its original parent II-*d* strain.

A similar trend induced by *in vitro* methods with a series of eleven strains representing -R, -*d*, and -*c* forms is shown in text-figure 4. In charting these results only those forms isolated *in vitro* have been recorded, and no attempt has been made to trace the dissociative patterns of each individual experiment. It will be seen from text-figure 4 that the major reversion trend is again in the direction of -*b* forms. Prior to the first acquisition of mouse virulence occasional forms, probably unstable, appear which give -S or -*a* types of agglutination. Subsequent to the first acquisition of mouse virulence, most of the S and -*a* forms maintain their virulence, but on the whole the organisms tend to stabilize themselves at the level of -*b* forms and not as the typical S and -*a* forms which we are accustomed to find in human or animal infections by the pneumococcus.

Another feature not very well shown in text-figures 3 and 4, is that when reversion of R, *-d* or *-c* forms takes place in mice; this *in vivo* reversion is apt to represent a rapid "ascent" to an S or *-a* form and whether intermediates actually appear in the mouse during the reversion process is not known. At least they were not isolated in these experiments although a careful search for them was not made.

COMMENT

Emphasis has been laid upon conditions which give rise to the development of forms intermediate between S and R pneumococci. These forms probably represent organisms endowed with varying amounts of S substances, or potentialities to develop S substances. They may appear progressively in the S → R transitions, and in reverse order in the R → S transitions described in this paper. Although symbols, based on priority of usage, have been employed to describe phases of the dissociative patterns, it has not been the major aim of this paper to classify rigidly the variants which have arisen during the evolutionary processes described. We have attempted rather to illustrate certain biological trends which occur in the pneumococcus species under certain conditions. Furthermore as the nature of these dissociative processes or trends is probably complex, still relatively obscure, involving as it does certain unsolved problems concerning so-called bacterial life cycles, and as we cannot evaluate the influence which the hypothetical presence of bacteriophage may have played, the attempt will not be made to discuss the mechanism of the processes involved. It would seem, nevertheless, as if the dissociative patterns above described have been limited, or influenced to some extent by the environment in which they have been produced. Our interpretation of this latter phenomenon is that the pneumococcus seems to emerge from a given environment in the form most fitted to survive in that environment. This concept renders a somewhat philosophical value to the question as to what form actually represents the true parent strain of the pneumococcus species, and this point will be elaborated in the following paper.

SUMMARY

1. The importance of recognizing intermediate forms which occur during *in vitro* transitions of S → R and R → S pneumococci has been stressed as a method for the study of dissociative processes in this species.

2. Two different methods of inducing degradative dissociation in S forms seem to give rise to two different patterns of variant production. Thus when S forms are grown in homologous antisera they become rapidly stabilized as R forms, but when S forms are grown in media containing bile, they show a greater tendency to become stabilized as *-c* forms.

3. During reversions of *-c*, *-d* and R forms, induced by growth in anti-R or plain rabbit serum broth, intermediates have been shown to arise in the reverse order to that in which they arise during the degradation of S forms, although such strains tend to become stabilized as *-b* forms, which is the usual "high" level to which these strains revert by this method.

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PLATE

PLATE 1

The colonies shown in figures 1 to 9 are 36- to 48-hours old unless otherwise specified. All but figures 1 and 7 represent a magnification of about 25 diameters.

FIG. 1. II-S colony (×20), dome shaped with smooth surface. Autolysis has not yet begun.

FIG. 2. I-*a* colonies. Flat disc shaped colonies in which autolysis has begun. The surface is faintly granular.

FIG. 3. II-*b* colony. After incubation for forty-eight hours this colony was left at room temperature for ten days. Some autolysis has taken place. The surface is faintly granular.

FIG. 4. II-*c* colonies. They are disc shaped, the surface is almost smooth, but granular about the periphery. The slightly elevated center is often a characteristic of intermediate and rough colonies.

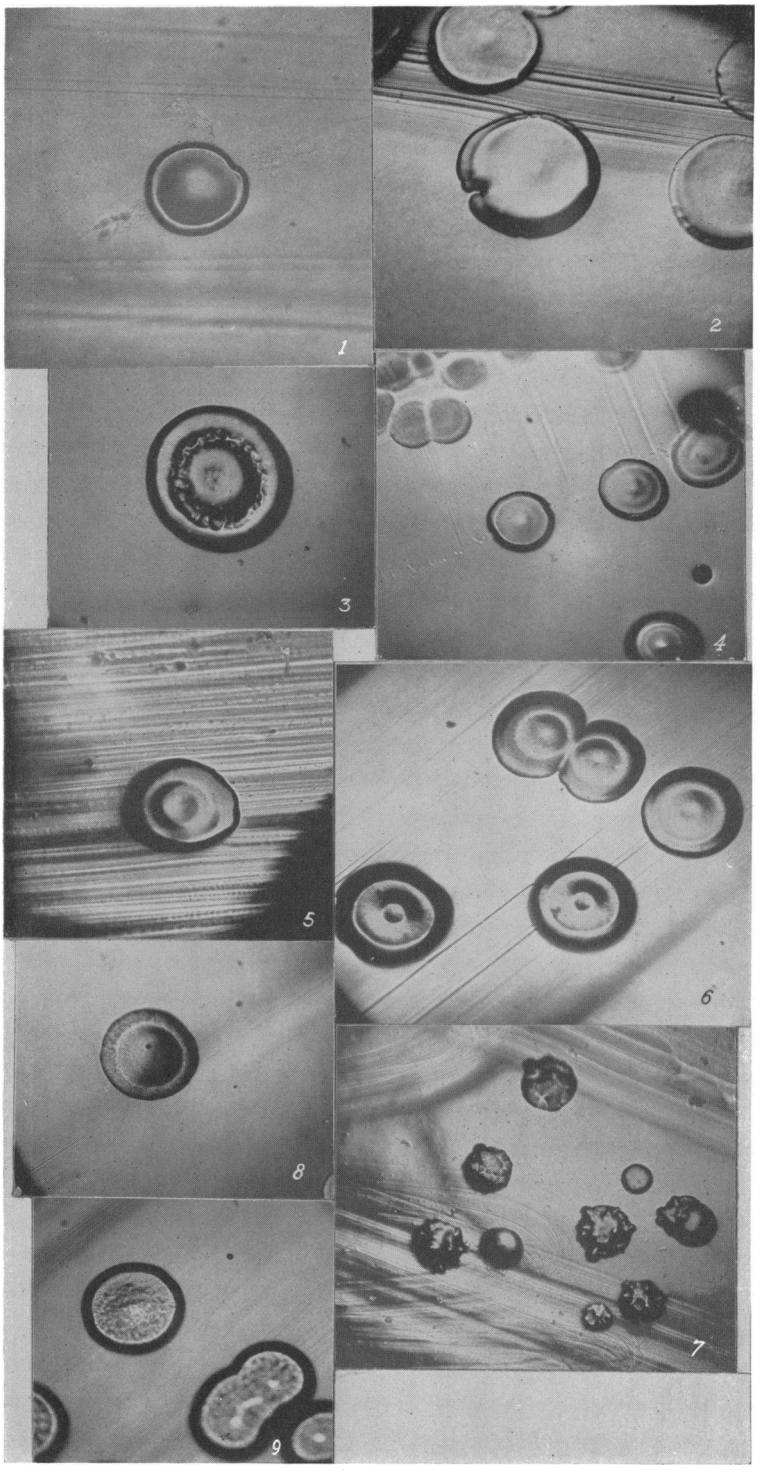
FIG. 5. A II-*c* colony. The surface is fairly smooth; slight central autolysis is present.

FIG. 6. II-*c* colonies.

FIG. 7. A mixture of II-*d* and II-R colonies (×15), representing a strain dissociating in media containing bile. The colonies were grown for 48 hours in the incubator and then stood for three days at room temperature. The II-*d* colonies did not autolyze. The II-R colonies have become irregular with daughter colony formation.

FIG. 8. A rather typical II-R colony.

FIG. 9. II-R colonies.



(John R. Paul: Pneumococcus variants)