STUDIES ON NATURAL IMMUNITY TO PNEUMOCOCCUS TYPE III

II. Certain Distinguishing Properties of Two Strains of Pneumococcus Type III Varying in Their Virulence for Rabbits, and the Reappearance of These Properties Following R→S Reconversion of Their Respective Rough Derivatives

By MORRIS F. SHAFFER,* Ph.D., JOHN F. ENDERS, Ph.D., and CHAO-JEN WU,* M.D.

(From the Department of Bacteriology and Immunology, The Harvard Medical School, Boston)

Plate 20

(Received for publication, May 11, 1936)

In the foregoing paper (1) it has been shown that while strains of Pneumococcus Type III incapable of multiplication at 41°C. are avirulent for the rabbit, not all strains able to persist and to grow at this temperature are equally lethal for that animal. Two smooth strains of the latter group exhibiting a striking disparity in their rabbit virulence have been studied more closely, and evidence obtained which indicates that the variation in virulence among such strains may depend at least partly on differences in size of the capsule and the rate at which this structure is lost.

The findings suggested an inquiry as to whether the capsular characteristics depend on factors which can be varied in the manner in which it has been demonstrated by Griffith (2), Dawson (3) and others that type specificity can be altered, or whether they might be the result of more stable physiological processes, which, although temporarily inapparent during existence in the R phase would again reappear unchanged on the resumption of the S form, regardless of whether the

* At the time this work was done Dr. Wu held a Travelling Fellowship from the Peiping Union Medical School, and Dr. Shaffer a Fellowship in the Medical Sciences from the National Research Council.

conversion was effected in the presence of killed smooth organisms from cultures of the rabbit virulent or avirulent strain. Below are presented the methods and the results of the experiments designed to answer these questions.

Technique

Cultures.—The two cultures, SV and CH, studied throughout the course of this work have been previously described (1). Rabbits survive the intravenous injection of 10 cc. of a 14 hour dextrose serum broth culture of CH, while the injection of 0.1 to 0.01 cc. of SV will bring about the death of the animal. Since CH grows but not as well at 41°C. as at 37°C., the experiments have been carried out at 37°C. to eliminate the factor of unfavorable influence of the higher temperature. Although the experiments reported here have been limited to only two of the strains studied in our first paper, our observations suggest that the findings apply also to the other strains.

Media.—The fluid medium adopted as standard for use throughout the experiments consisted of 100 cc. of sterile infusion broth to which was added 1 cc. of a sterile 10 per cent solution of dextrose in saline, together with 0.5 cc. normal rabbit serum. This medium was inoculated with 1 per cent of its volume of a 16 hour rabbit blood hormone broth culture, seeded with material from the stock cultures maintained in the manner already noted. In the study of colonial morphology, the horse blood neopeptone glycerin agar recently described by Ward and Lyons (4) was used.

Preparation of Antisera.—No great difficulty was encountered in producing in rabbits antisera of adequate titre against Pneumococcus Type III. 18 hour cultures in 0.1 per cent dextrose infusion broth of the strains CH and SV were centrifuged and the bacterial deposits taken up in a volume of 0.3 per cent formalin in saline equivalent to one-half that of the discarded supernatant, allowed to stand for 72 hours at 37°C. and then tested for sterility. Immunization of rabbits was carried out according to the method of Cole and Moore (5), using as the daily dose 0.5 cc. of vaccine. It was usually found necessary to administer three to five weekly courses of injections before securing a sufficient quantity of type specific agglutinin and precipitin. The anti-R sera were obtained in the same manner, using a rough variant derived from Pneumococcus Type I, with the exception that the vaccine was prepared without the use of formalin by heating the organisms for 20 minutes at 56°C.

Agglutination and Absorption of Agglutinins.—Unless a different procedure is described in the text, the antigens for agglutination were prepared by centrifuging the organisms from 15 hour broth cultures and resuspending the deposit in the original volume of fluid with 0.3 per cent formalinized saline. In testing for agglutinins, equal portions of the bacterial suspension and serum dilutions were mixed, left for 2 hours at 37°C. and overnight in the ice box before reading.

In the experiment demonstrating the absence of a distinctive agglutinogen in

strain SV, 0.5 cc. quantities of the antisera used were twice absorbed with the deposit from 100 cc. of 0.1 per cent dextrose infusion broth culture, allowing a period of exposure to each absorbing dose of 1 hour at 37°C. 0.5 cc. of the antisera employed in demonstrating the similarity of old cultures of CH and SV to R variants in their agglutinative behavior were absorbed twice with the deposits from 20 cc. of 5 and 10 hour dextrose serum broth cultures of CH and the R variant respectively.

Capsule Stain.—After trial of many of the capsule stains in general use, none of which were entirely satisfactory, the following stain was devised. Organisms either from culture or the animal body are emulsified on the slide in a drop of normal rabbit serum. The air-dried unfixed preparation is then stained for 1 minute with an alcoholic solution of methyl violet (to 1 part saturated solution of methyl violet in ethyl alcohol filtered through paper is added 1 part of ethyl alcohol). The excess stain is shaken off and the slide flooded with an alcoholic solution of basic fuchsin (to 1 part saturated solution of basic fuchsin in ethyl alcohol filtered through paper is added 4 parts of alcohol). This stain is allowed to act for 20 seconds and not longer. The preparation is then washed very rapidly in water and immediately blotted dry.

Method Used in Determining Relative Size of Organisms.—Calibrated Hopkins centrifuge tubes were employed, in a method similar to that of Jones and Little (6) in their study on increase in size of bacteria after sensitization with antiserum. Using the same cleaned pipette for all determinations, equal volumes (usually 0.9 cc.) of the various cultures were placed in the Hopkins tubes and centrifuged at 2800 R.P.M. for half an hour. The length of the column of bacteria deposited was measured to the nearest 0.1 mm. with a square jawed micrometer caliper, placing a plane mirror behind tube and caliper to obtain greater accuracy in reading. It was found that the ratio between the lengths of bacterial deposits determined after centrifuging for half an hour remained practically unchanged, even when centrifuging was continued for 45 minutes and 1 hour. Chilled samples of the cultures were diluted with a solution of methylene blue (0.1 cc. saturated alcoholic solution of the dye in 10 cc. saline) and counted in a bacterial counting chamber. The bacteria in 100, 1/400 mm. squares were enumerated. From the data thus obtained the relative volumes of equal numbers of organisms of the cultures to be compared were calculated. Duplicate determinations made on the same day, using the same culture, usually agree closely. Occasionally, measurements made on different days may show appreciable variation, probably due to the inherent difficulty of obtaining identical conditions of growth in different experiments. In order to minimize such discrepancies, the average of several determinations at each time interval has been taken.

Acid Agglutination.—Dextrose serum broth cultures of varying ages were centrifuged and the deposits taken up in sufficient saline to give whenever possible approximately the same density of suspension. To 0.25 cc. of a series of buffers varying from pH 1.1 to pH 6.0 was added 0.1 cc. of suspension. Sörensen's glycocoll-hydrochloric acid buffers were used for the range pH 1.1 to pH 2.3, and

McIlvaine's disodium phosphate-citric acid mixtures for the range pH 2.2 to pH 6.0. The pH of each buffer was checked potentiometrically. After being left 30 minutes at 37°C. and overnight in the ice box, readings were taken.

Phagocytosis.—The procedure employed in experiments involving the use of polymorphonuclear leucocytes in normal adult human defibrinated blood was essentially that described by Ward and Enders (7). Broth cultures were centrifuged and the sediment resuspended in sufficient broth to concentrate the numbers of pneumococci severalfold. In phagocytic experiments involving the use of rabbit leucocytes and serum the technique given by Enders and Wu (8) was adopted.

Method of Producing the R Variants.—1 drop of 18 hour blood broth cultures of CH and SV respectively was inoculated into 5 cc. of beef infusion broth containing 10 per cent antipneumococcus Type III rabbit serum (agglutinating titre 1/190 against 20 hour cultures of homologous strain CH) and incubated at 37°C. Thereafter, daily transfers were carried out in antiserum broth, using 1 drop as the inoculum. By the fifth transfer some R colonies were observed on plating out the cultures of both strains on 5 per cent horse blood infusion agar. All the colonies developing from the eighth transfer showed R characteristics. In each case, one colony was fished to blood broth. From the resulting cultures five daily transplants in this medium, as well as corresponding platings, were carried out; in none of the latter were S colonies observed. From the fifth such transfer single colonies were again fished and these cultures (designated CH-R and SV-R) of the two strains preserved for use. 0.7 cc. of either R culture was not fatal to mice on intraperitoneal injection.

EXPERIMENTAL

Cultural Characteristics

Growth in Fluid Media.—From data obtained by plating serial dilutions of samples taken at intervals from both CH and SV cultures when grown in dextrose serum broth at 37°C., it was possible to construct growth curves. These (Text-fig. 1) showed that the duration of lag and rate of multiplication in the logarithmic growth phase were nearly the same for these two strains. The time at which the maximum was reached was slightly later and the maximum numbers some 8 to 10 times less in the case of SV than for CH, both in dextrose serum broth and in blood broth. In defibrinated rabbit blood at 37°C. similar curves were obtained, with the maximum numbers for each being approximately equal. We wish to emphasize these similarities as being of basic importance since they indicate that the differences described in succeeding sections are not merely apparent and due to any prolonged lag of SV but represent real attributes which form distinguishing characteristics between the two strains even when they are in the same phase of growth.

The two R forms also exhibit distinctive properties when grown in blood broth. Here CH-R produces a diffuse clouding of the medium in contrast to the formation

of moderate sized granules by SV-R. If rapid transfers of the latter are carried out, it can be induced to grow diffusely; nevertheless, when the diffusely growing culture is plated on horse blood agar and again cultivated in broth, the granular type of growth is resumed. The SV-R growth in broth is likewise less luxuriant than that of CH-R.

Growth on Solid Media.—The two smooth strains cannot be differentiated on the usual 5 per cent horse blood infusion agar, since both form the large mucoid lenticular colonies typical of Pneumococcus Type III. On the neopeptone glycerin horse blood agar of Ward and Lyons after 16 hours, the CH colonies appear definitely whiter in comparison with the delicate pearl grey of SV. After 44 or more hours of incubation the flat CH colonies show dull granular surfaces with multiple small shiny prominences resembling tiny daughter colonies, contrasting with the dull smooth surface of SV colonies which is only rarely broken by a small papular eminence. The rough variants differ when grown on the latter medium. At 20 to 24 hours, CH-R is distinguished by a central portion much rougher in appearance than that of SV-R. After 48 hours the CH-R colonies (1.25 mm. diameter) are low convex or flat in shape. The surfaces, which fail to reflect the light, are covered with multiple papillae; the edges are smooth. At this time, optimal for differentiation, the slightly smaller SV-R colonies (1 mm. diameter) are slightly conical in shape, with a finely granular surface and irregular edges.

Evidence for the Identity of Antigenic Structure

Before proceeding further we considered it necessary to obtain evidence of the antigenic identity of the two strains. To this end, portions of antisera prepared against CH and SV were absorbed with the homologous and heterologous strain. To dilutions of these, as well as the unabsorbed sera, formalinized saline suspensions of the two strains were added. The results showed that there occurs mutual cross-absorption of the agglutinins produced in response to the injection of either organism into rabbits. Although this experiment does not completely eliminate the possibility of the existence of a distinguishing antigen situated deeply within the organism, it indicates at least that the more superficial antigenic layers primarily concerned in rendering the organism invulnerable to the defenses of the host are identical for the two strains.

Variation in Size of Capsule during Growth

The size and character of the CH and SV capsules were studied in stained smears during the first 34 hours of growth in broth at 37°C. From an examination of the series of photographs of such preparations reproduced in Figs. 1 to 20 it will be seen that after 3 to 4 hours incubation both strains possess well developed

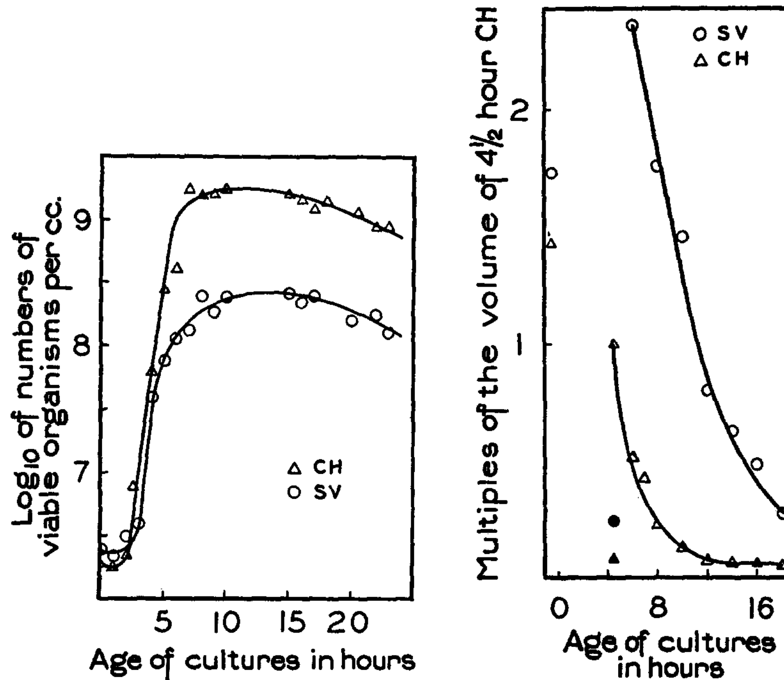
capsules, whereas the organisms in the original inoculum were noncapsulated. The envelope surrounding CH cells is, however, in most cases, somewhat smaller and does not stain so well or appear so dense as that of SV. On further incubation, the CH capsule becomes rapidly reduced and after 7 to 8 hours nearly all of the organisms have no stainable capsular material, although a few may still retain traces. At 10 hours no vestige is demonstrable. On the other hand, the majority of the individuals of SV retain even after 14 hours very large capsules with a periphery which takes the stain well. By 16 hours increasing numbers of decapsulated forms are seen, but it is not until 24 hours that practically every coccus appears devoid of capsule. We have noted that the dimensions of the cocci exclusive of the capsule are slightly greater in young than in old cultures, but have not studied this aspect of changes in size more closely.

We have also followed by means of stained smears the changes occurring when the two strains are grown at 37°C. in fresh normal rabbit serum or defibrinated rabbit blood, by inoculating 3 or 4 cc. of either of these media with 1 per cent of its volume of a 16 hour blood broth culture. In serum the CH cocci tend to grow in short chains, around which narrow faintly staining capsules are first definite after 3 hours, while the SV organisms appear chiefly as diplococci with large intensely stained capsules even after 1 to 2 hours. After 6 hours incubation only faint traces of fuzzy and uneven capsular material are observed in CH cells, while the SV organisms still show relatively large intact capsules after 13 hours. In blood the results are similar.

Since observed differences by the smear method might be exaggerated by artefact and, since through its use quantitative information concerning the relative size of the organisms could not be readily obtained, the results were confirmed and extended by measurement of the volume occupied by the bacteria at different times during growth. The relative sizes have been determined: (*a*) by comparing with 4½ hour CH as standard, the volumes occupied by equal numbers of the two strains grown in dextrose serum broth for periods varying usually by 2 hour intervals; (*b*) by ascertaining the ratio of the volume occupied by SV organisms to that occupied by equivalent numbers of CH of the same age during a period of incubation extending from 6 to 18 hours.

It has been impractical to secure determinations of the volume of CH before 4½ hours or SV before 6 hours, owing to the paucity of growth, while after 18 hours a tendency to clumping makes it difficult to enumerate the organisms in the bacterial counting chamber. From Text-fig. 2 it will be seen that the average volume of the SV cocci at 6 hours is 2.5 times that of CH at 4½ hours. Were it possible to secure sufficient numbers of each strain at a still earlier period this difference might possibly be even less, for on the basis of the smears we know that by 4½ hours many of the CH organisms have lost some of their capsules. There is no reason to believe, however, that strain CH ever quite attains the size of SV, since, as is shown by the points to the left of the curves in Text-fig. 2, even the volume of CH organisms removed from the peritoneal cavity of the mouse killed 20 hours after infection is somewhat less than the volume of SV similarly obtained. Further

examination of this figure shows a rapid diminution in the volume of both organisms during the 6 to 12 hours incubation interval, with the average volume of CH after 10 to 12 hours having reached that of its R variant at 4½ hours. In contradistinction, SV originally characterized by a larger capsule, requires more than 18 hours of growth to approximate the volume of its R derivative which at 4½ hours is three to four times as large as CH-R.



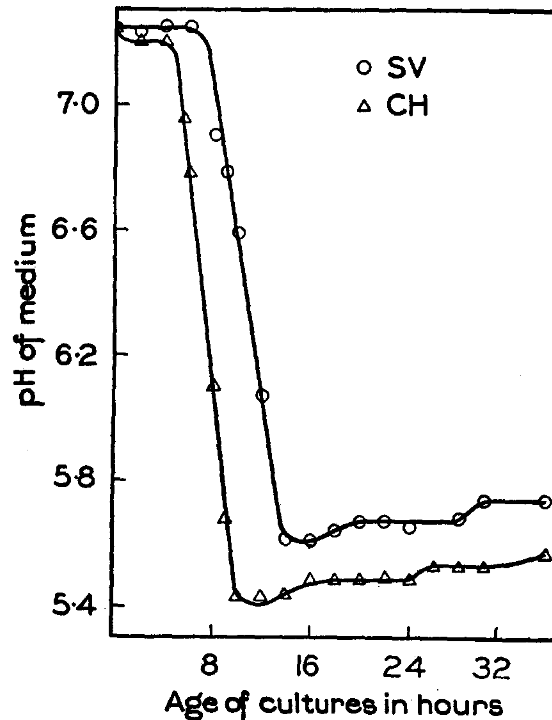
TEXT-FIG. 1 TEXT-FIG. 2

TEXT-FIG. 1. Growth curves of Pneumococcus Type III, strains CH and SV, at 37°C. in dextrose serum broth.

TEXT-FIG. 2. A comparison of the relative volumes of Pneumococcus Type III, strains CH and SV, in broth cultures of varying age with the volume of the organisms in a 4½ hour culture of strain CH taken as unity. At the lower left are also indicated by solid black the volumes of the respective R variants at 4½ hours. Points to the upper left of the curves indicate relative volumes of pneumococci taken from the peritoneum of the mouse.

In interpreting these results we consider that the alterations in volume represent chiefly changes in the quantity of capsular material

retained because of their agreement with the evidence offered by stained preparations and the fact that both encapsulated organisms ultimately are reduced to approximately the volume of their respective R forms which at 6 hours is about one-eighth that of the smooth cultures of the same age. Furthermore, the volume of CH after 10 to 12 hours undergoes no further diminution, as might be expected to occur



TEXT-FIG. 3. Changes in pH of medium during growth of Pneumococcus Type III, strains CH and SV, in broth cultures.

if the soma were shrinking, but remains practically unchanged in size. The disparity in size between the two R forms, which may also be demonstrated in stained preparations, is apparently not associated with any structure analogous to a capsule but is dependent upon the possession by SV-R of a greater quantity of somatic material.

We thought it possible that the early loss of CH capsule might be effected by a greater and more rapid increase in the acidity of the

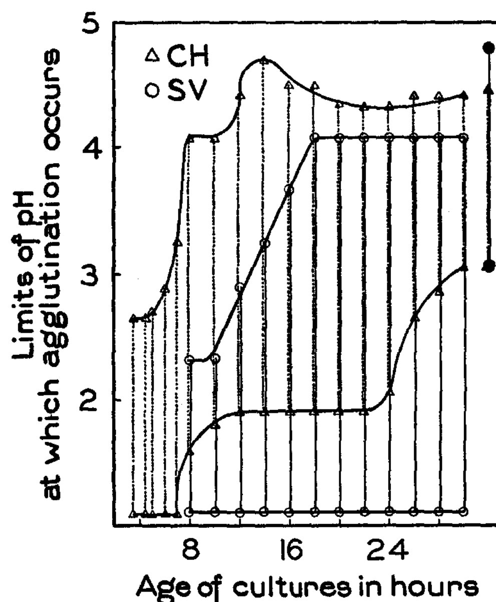
medium. Accordingly, potentiometric measurements of the hydrogen ion concentration were made on the culture medium at frequent intervals and curves constructed from the data. From Text-fig. 3 it is seen that in the CH culture the pH does fall sooner and reaches a slightly lower level. That the more rapid loss of capsule does not, however, depend on this increased acidity is demonstrated not only by the fact that the lowest pH is attained by SV at a time when it still possesses large capsules (12 hours), but also by experiments in which the reaction of the cultures was maintained at pH 7.2 during the same growth period through hourly additions of N/1 NaOH. No difference in capsular dimension was noted between smear preparations from the alkalinized cultures and untreated controls. Furthermore, experiments have been performed in which cultures were acidified with N/1 HCl to pH 5.4 at a time when the organism possesses abundant capsule (after 4 hours incubation for CH; either 4 or 7 hours for SV). Smears were then made from time to time during continued incubation. It was found that even after 24 hours the acidified cultures had not lost their intact well developed capsules while the untreated controls showed none. Thus the presence of acidity equivalent to that developed during growth not only did not dissolve the capsular material but appeared to favor its retention. Although we have not been able to eliminate other unknown factors in the environment which might account for the difference in the rate of capsular loss, we believe on the basis of the available evidence that this is an inherent function of the individual strain.

Acid Agglutination at Various Periods

The striking diminution in volume led to the thought that definite alteration in the surface of the organisms should take place attendant upon the loss of capsular material. Thus substances of different chemical composition might be exposed which could shift the range of acid agglutination characteristic of the completely capsulated organism toward that in which clumping of the noncapsulated R variants occurs. Such displacement should be observed sooner in the case of CH.

In subjecting this hypothesis to experiment, cultures of CH and SV of varying age were centrifuged, resuspended in saline and added to

a series of buffers. Young cultures of the R variants of the two strains were likewise tested. The results are assembled in Text-fig. 4 in the form of a graph. It will be seen that in cultures of CH not more than $3\frac{1}{2}$ to 5 hours in age is the range of acid agglutination from pH 1.0 to pH 2.65, which corresponds to the range exhibited by SV even at 10 hours, except that the CH zone is slightly broader. We have found that fully capsulated organisms of both strains taken



TEXT-FIG. 4. Changes in the zone of acid agglutination during growth of broth cultures of Pneumococcus Type III, strains CH and SV. Solid and dotted vertical lines represent ranges of acid agglutination of strains SV and CH respectively. To the right of the curves are represented in solid black the zones for the R variants.

from the mouse peritoneum also agglutinate in the range pH 1.1 to pH 2.8. Beginning after 6 hours a swift displacement of the agglutinative range of CH occurs, which by 12 hours extends from pH 1.9 to pH 4.4 and which remains unchanged during the 10 hours following, except for slight fluctuations at the alkaline end. At 24 hours a second change begins at the acid end, causing a gradual contraction of the agglutination zone which at 30 hours becomes identical with that found for the homologous R form at $4\frac{1}{2}$ or 7 hours.

The behavior of strain SV is quite distinct. No change occurs until 12 hours when an extension of the alkaline end begins which takes place at a slower rate than the earlier rise of CH and attains a maximum only after 18 hours. Agglutination is found on the extreme acid end throughout the entire period of observation but although always marked, it may be incomplete in tubes of pH 1.1 to 2.0 after 16 to 18 hours or more of growth. The pH of the most alkaline buffer in which SV agglutinates is somewhat lower than that determined for the $4\frac{1}{2}$ or 7 hour homologous R form.

The use of buffers of pH as low as 1.1, which have not usually been employed in previous studies of acid agglutination, has revealed important differences in the behavior of the two strains. Gillespie (9) in his work on the acid agglutination of Pneumococcus Type I and III used lactate and acetate buffers ranging from pH 2.4 to pH 5.4 with cultures of 18 and 42 hours. He noted differences between these cultures in the hydrogen ion concentration producing agglutination. Hughes (10) also studied the agglutination of 8 hour cultures of typical and variant pneumococci in the buffer mixtures of Northrop and De Kruif, but observed no clumping of Pneumococcus Type III below pH 2.7. Presumably his culture was similar to CH. Sherman and Albus (11) found no agglutination of 4 hour cultures of *B. coli* in buffers of pH 3.0 to 4.1 to which the corresponding 24 hour cultures were susceptible.

Although Gillespie found that repeated washing does not appreciably alter the results, we have observed that with $4\frac{1}{2}$ to 6 hour CH cultures or suspensions from the mouse peritoneum, organisms washed one or more times with saline no longer agglutinate from pH 1.1 to 1.9, while SV organisms even in cultures as old as 16 hours remain unaffected by this procedure.

In all likelihood agglutination occurring from pH 1.1 to pH 2.3 or 2.6 is due to the material at the exposed surface of the capsule. Since the specific polysaccharide is an important constituent of the capsule it is not illogical to think that it may be the substance upon which this agglutination mainly depends. We have tested a sample of Pneumococcus Type III specific soluble substance, purified according to the procedure of Heidelberger, Goebel and Avery (12), by adding 0.1 cc. of an 0.08 per cent solution to a series of buffer tubes in the same manner as for acid agglutination. It was found that this SSS was thrown out of solution in the buffers of pH 1.1 to pH 1.5. A preparation of the

TABLE I

Agglutination of Strains SV, CH and a Rough Variant of Pneumococcus Type III in Type Specific and Anti-R Sera before and after Absorption

Strain	Antiserum	Dilution of antisera							Remarks
		1/3	1/6	1/12	1/24	1/48	1/96	1/192	
CH 6 hrs.	Anti-S	−	+++±	++++	+	0	0	0	Large flakes
	Anti-S absorbed with CH	−	0	0	0	0	0	0	
	Anti-S absorbed with R III	−	+++	++++	++	±	0	0	Large flakes
	Anti-R I	0	0	0	0	0	0	0	
CH 20 hrs.	Anti-S	−	++++	++++	++++	++++	++++	++	Medium sized flakes
	Anti-S absorbed with CH	−	0	0	0	0	0	0	
	Anti-S absorbed with R III	−	++++	++++	++++	++++	+++	++	Medium sized flakes
	Anti-R I	+++	+++	+	±	0	0	−	Small granules
CH 30 hrs.	Anti-S	−	++++	++++	++++	++++	++	+	Small granules
	Anti-S absorbed with CH	−	0	0	0	0	0	0	
	Anti-S absorbed with R III	−	++++	++++	++++	++++	±	0	Small granules
	Anti-R I	++++	+++	++	−	±	0	−	Small granules
SV 8 hrs.	Anti-S	−	++++	+++±	±	0	0	0	Large flakes
	Anti-S absorbed with CH	−	0	0	0	0	0	0	
	Anti-S absorbed with R III	−	++++	+++	±	0	0	0	Large flakes
	Anti-R I	+	±?	0	0	0	0	−	
SV 20 hrs.	Anti-S	−	+++±	+++±	+	0	0	0	Large flakes
	Anti-S absorbed with CH	−	0	0	0	0	0	0	
	Anti-S absorbed with R III	−	++++	+++±	+±	±?	0	0	Large flakes
	Anti-R I	±	±	0	−	0	0	−	
SV 30 hrs.	Anti-S	−	++++	++++	++++	+++	0	0	Small granules
	Anti-S absorbed with CH	−	0	0	0	0	0	0	
	Anti-S absorbed with R III	−	++++	++++	++++	+	0	0	Small granules
	Anti-R I	++++	+++	+++	±	0	0	−	Small granules
R III 24 hrs.	Anti-S	−	++++	++	±	0	0	0	Small granules
	Anti-S absorbed with CH	−	±	0	0	0	0	0	
	Anti-S absorbed with R III	−	±	0	0	0	0	0	
	Anti-R I	++++	++++	+++	++	0	0	−	Small granules

Readings after one hour at 55°C. and overnight in the ice box.

+ = agglutination.

0 = no agglutination.

− = not done.

± = trace of agglutination.

R III = the R form derived from strain SV. Anti-S = antipneumococcus Type III rabbit serum. Anti-S absorbed with CH = anti-S serum absorbed with organisms from a 5 hour dextrose serum broth culture of strain CH. Anti-S absorbed with R III = anti-S serum absorbed with a 10 hour dextrose serum broth culture of the R variant derived from strain SV. Anti-R I = antipneumococcus rabbit serum prepared by injection of an R variant obtained from Pneumococcus Type I.

specific carbohydrate purified in a different manner (Hornus and Enders (13)) precipitated only slightly in this pH range and then only after 2 to 3 days. We believe, however, it is the type specific substance upon which agglutination under these conditions depends and that the loss of the greatest proportion of its capsular substance by CH may account for the failure to agglutinate in buffers of pH 1.1 to 1.7 after 10 hours cultivation, whereas enough individuals of strain SV retain sufficient quantity of this material for as long as 20 to 30 hours to remain agglutinable in these concentrations of hydrogen ions.

If the curves for the acid agglutination values are compared with those shown in Text-fig. 2 it will be evident that the rapid loss in CH volume takes place synchronously with an equally rapid shift in the agglutinating range toward a more alkaline pH. Extension of the zone of SV agglutination in the direction of increased alkalinity begins only when the volume is reduced to an amount equivalent to that of CH at the time the latter exhibits an analogous agglutinative shift.

Agglutination in Type Specific Antiserum and Anti-R Serum

It seemed reasonable to think that the marked changes in susceptibility to clumping in buffers as growth proceeds denoted altered physicochemical properties of the bacterial surface which should be manifest when antigen was exposed to antibody. In Table I are recorded the results of an experiment in which the agglutinative behavior of living saline suspensions of both strains at different stages of cultivation was observed in the presence of anti-type specific and anti-R sera. They show that 6 hour cultures of CH which agglutinate in the anti-Type III pneumococcus serum fail to do so in anti-R serum, while 20 or 30 hour cultures are agglutinated in the latter serum to approximately the same titre as the R form. Nevertheless these older cultures are also clumped by type specific agglutinin, since absorption of the type specific antiserum with R organisms fails to remove this agglutinin which is completely taken out by a 5 hour culture of CH. Type specific agglutination in old cultures appears to be due to the very small quantities of the specific soluble substance too minute to greatly affect the zone of acid agglutination, but capable of uniting with homologous antibody. Marked reduction in the amount of type specific capsular material per organism also may account for the fact

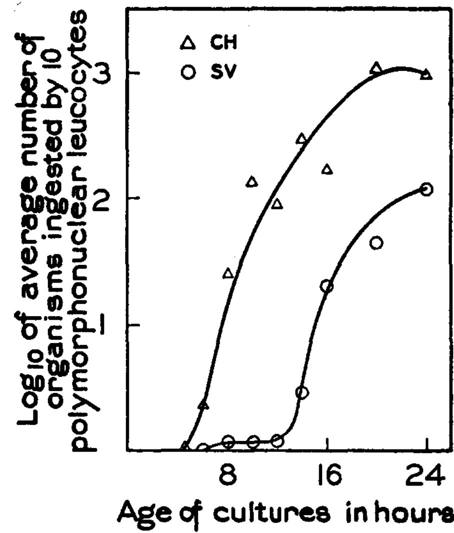
that the agglutinin titre of the anti-S serum against CH is about eight times greater if the antigen consists of a 20 or 30 hour culture than if a 6 hour culture is employed. We have confirmed this quantitative difference in the behavior of young and old cultures in similar experiments in which suspensions containing equal numbers of organism were used.

In general, SV agglutination in the various sera follows that of CH, but the changes occur at a later period. Thus in anti-R serum no definite agglutination is seen in 20 hour cultures. At 30 hours, however, in the anti-R serum it is agglutinated nearly to the titre of its homologous R derivative. No displacement of the end-point of agglutination in anti-type specific serum occurs with the 20 hour cultures while even with a 30 hour culture the titre of agglutinin appears only to be doubled in contrast to the apparent eightfold increase with the 20 hour CH culture. This disparity in the quantity of type specific agglutinin necessary to bring about equivalent effect in older cultures of SV may well be related to the hundred- to thousandfold difference noted by Watson and Cooper (14) in the quantity of protective substance required to save mice infected with the same doses of a rabbit virulent and a rabbit avirulent strain of Pneumococcus Type III. Finally we wish to point out that although a 6 hour CH culture fails to agglutinate in anti-R serum, it is nevertheless capable of binding agglutinin reacting with the R variant. Pertinent in respect to these findings are those of Tillett (15) who showed that chemically decapsulated Pneumococcus Type III agglutinated to the same titre as the R variant in anti-R serum.

Phagocytosis by Human Polymorphonuclear Leucocytes

The readiness with which strains CH and SV were taken up by the polymorphonuclear leucocytes in normal adult human defibrinated blood was determined for dextrose serum broth cultures of varying age. From Text-fig. 5 it will be observed that $4\frac{1}{2}$ hour cultures of strain CH are completely resistant to phagocytosis. As early as 6 hours a few organisms have reached a state such that they can be ingested. Thereafter a rapid rise in the number of intracellular cocci takes place. Sharply at variance with this swift loss of resistance to phagocytosis is the prolonged retention of the property by SV. Very few organisms less than 14 hours in age appear in leucocytes,

but at this time a slight increase in phagocytosis is observed which becomes greater with further cultivation. In the following paper observations concerning the phagocytosis of these strains by the leucocytes of the rabbit and mouse have been recorded. We may note here, however, that in a system of normal rabbit leucocytes and serum, active phagocytosis of both R variants occurred. In one experiment the number of cocci from a 6 hour CH-R culture ingested by ten polymorphonuclears was 385, while 108 SV-R organisms were



TEXT-FIG. 5. Phagocytosis by polymorphonuclear leucocytes in normal adult human defibrinated blood of Pneumococcus Type III, strains CH and SV, at intervals during growth in broth.

taken up by the same number of cells. Since in each case the leucocytes appeared to be completely filled with organisms, this difference may not represent any greater susceptibility to phagocytosis of CH-R, but probably depends on the greater somatic volume per coccus of SV-R.

Reconversion of the R Variants to S Forms

Employing the method of Griffith, the two R strains possessing the characteristics described above were converted to the S form under the following conditions: (*a*) injection into mice of a small quantity of

the living R form derived from either the rabbit virulent or avirulent strain, together with a large quantity of heat-killed S form of the homologous or heterologous strain; (*b*) injection of a large quantity of the living R form alone.

The killed S vaccines were prepared by centrifuging the deposits from 6 hour dextrose serum broth cultures of smooth strains CH and SV and resuspending in sufficient physiological saline to yield suspensions concentrated about 150-fold, which were heated at 60°C. for ½ hour. Their sterility was controlled by inoculation of plates and broth as well as mice.

White mice weighing 15 to 18 gm. were injected subcutaneously with 0.5 cc. of these vaccines together with 0.5 cc. of a 15 hour blood broth culture of the R variant. Additional mice were injected subcutaneously with 0.5 cc. of broth suspension of the R variants representing the centrifuged deposit from 100 cc. of 15 hour blood broth culture. The combined details and results of two experiments are recorded in Table II.

From the cultures of the heart's blood of the mice dying as a result of the infection, typical smooth colonies were fished to blood broth and studied in respect to colony morphology on the Ward-Lyons medium, growth characteristics in broth, survival in defibrinated rabbit blood under vaseline seal at ice box temperature, capacity to resist the phagocytic action of normal human leucocytes and serum, and finally the virulence of certain selected ones for the rabbit.

Appearance of Colony Produced by Reconverted S Forms and Characteristics of Growth in Broth.—Broth cultures developing from the single colonies fished from the platings of the heart's blood of the mice were seeded onto the glycerin blood agar medium along with organisms from cultures of the stock strains of CH and SV. A careful study of the single colonies showed that in every case in which living SV-R had been injected either alone or together with heat-killed S, the colony morphology corresponded to that of the stock smooth SV, regardless of whether or not the vaccine (if employed) was prepared from this strain or from CH. On the other hand, in the animals which had received CH-R the smooth forms which developed appeared to be identical in respect to the colony with the stock CH even when produced under the influence of SV vaccine. From these observations, it is clear that when reversion to the smooth form took place the morphology of the colony was the same as that of the strain from which the R variant derived, and was not influenced by the origin of the vaccine employed as an agent in the process of conversion.

Colonies picked from the plate cultures used for the morphological study were inoculated into blood broth and the density of the growth noted. In this characteristic, also, the reconverted strains resembled the stock smooth strains, since cultures of organisms from reconverted CH-R, independent of the means used in effecting the transformation, with one exception grew luxuriantly, in contrast to those representing SV-R brought back to the S phase, all of which exhibited a more

delicate growth. In these studies 36 colonies were tested; two to six colonies were selected from the heart's blood culture of each mouse.

Survival of Reconverted R Variants in the Cold.—We had made the observation that under the conditions in which the stock strains were maintained (1), strain SV would survive for periods exceeding 18

TABLE II

Reconversion of R Variants of Pneumococcus Type III to the Smooth Form

Group	Materials injected	No. mice in each group	No. mice dying in each group and time of death	Results of cultivation of heart's blood	Results of cultivation of material from site of injection	Remarks
1	0.5 cc. CH vaccine	3	0	—	—	
2	0.5 cc. SV vaccine	3	0	—	—	
3	0.5 cc. CH-R culture	3	0	—	—	
4	0.5 cc. SV-R culture	1	0	—	—	
5	0.5 cc. CH-R culture + 0.5 cc. CH vaccine	5	2—72 hrs.	Many R and a few S colonies	Many R and a few S colonies	3 surviving mice killed after 2 wks. Cultures of heart's blood and injection sites were sterile
6	0.5 cc. CH-R culture + 0.5 cc. SV vaccine	5	3 {1—48 hrs.; 2—72 hrs.}	All S, no R colonies	Many S and a few R colonies except in one case which gave all S forms	Cultures from survivors were sterile
7	0.5 cc. SV-R culture + 0.5 cc. CH vaccine	1	1—60 hrs.	All S, no R colonies	Many S and a few R colonies	
8	0.5 cc. SV-R culture + 0.5 cc. SV vaccine	1	1—60 hrs.	All S, no R colonies	Many S and a few R colonies	
9	100 cc. CH-R culture	2	1—72 hrs.	All S, no R colonies	Not done	Cultures from survivor killed after 2 wks. were sterile
10	100 cc. SV-R culture	2	2 {1—72 hrs.; 1—96 hrs.}	1—S and R colonies; 1—only S colonies	Not done	

months, while CH under the same conditions died out within 9 months to a year. With this in mind, the viability of a selected number of the cultures was tested after the cultures in defibrinated rabbit's blood under a vaseline seal had remained in the ice box for 16 months. Eight cultures representing the various combinations involving the

transformation of SV-R and seven cultures originally obtained from mice injected with CH-R were plated. All of the former were found to be viable, whereas none of the reconverted CH-R forms had survived, nor a culture of CH stock kept under the same conditions. In this respect then the S forms obtained from CH-R and SV-R behaved like the original smooth cultures, although in certain instances their reconversion was mediated by vaccine derived from the heterologous strain.

Resistance to Phagocytosis of the Reconverted Strains.—Since it was found that there was a marked difference in the resistance of young cultures of the two stock smooth strains to the phagocytic action of normal human leucocytes, the effect of this system on 6 hour blood broth cultures of 32 of the 36 reconverted colonies noted above was studied. Upon testing these within 1 to 2 weeks following their transformation it was observed that in the case of cultures, emanating from both CH-R and SV-R that their resistance to phagocytosis was not identical with that of the smooth stock strains (Table III).

It is difficult to offer an explanation for this behavior. That it is however, at least in the case of the reconverted SV-R cultures, a transitory phase, representing possibly unstable properties on the part of some of the organisms which after a time become fixed according to the pattern of the stock strain, is demonstrated by the fact that after maintenance in defibrinated rabbit blood for 16 months in the ice box these, like the stock strain of SV, almost completely resist the phagocytic attack by the cells of the same normal human blood in which they were first tested. Since the cultures of reconverted CH-R colonies had died out during this time, it was impossible to determine their resistance to phagocytosis.

Virulence for Rabbits of the Transformed R Variants.—16 to 18 hour blood broth cultures obtained from certain colonies selected from those which were studied and which represent the various combinations utilized in transformation were injected intravenously into rabbits with the object of determining their virulence for those animals. The cultures of reconverted CH-R variants which were tested were those which were found to exhibit the greatest resistance to phagocytosis in the previous experiments. The experimental details are incorporated in Table IV. From those recorded for the first four animals it is

apparent that in every instance in which the rough variant was obtained from the rabbit virulent strain and again converted to the smooth form, the latter killed the rabbit, whatever the means employed for accomplishing the transformation. It thus appeared that the capacity of the transformed R variant of the rabbit virulent strain to produce in small amounts a fatal infection in rabbits was not

TABLE III

Phagocytosis in Human Blood of Organisms from 6 Hour Blood Broth Cultures of Reconverted and Stock S Strains

Source of culture	No. of colonies* studied	Average of phagocytic counts on cultures from colonies (No. cocci per 10 leucocytes)	Highest count (No. cocci per 10 leucocytes)	Lowest count (No. cocci per 10 leucocytes)	Remarks
Mice injected with CH vaccine and CH-R culture	6	45.4	88.5	15.5	3 colonies studied from each of 2 mice injected with this combination
Mice injected with SV vaccine and CH-R culture	9	51.2	82.5	20.5	3 colonies studied from each of 3 mice injected with this combination
Mouse injected with CH-R culture only	2	93.7	121.0	66.5	2 colonies studied from 1 mouse injected
Stock CH culture	—	74.9	—	—	
Mouse injected with SV vaccine and SV-R culture	6	24.6	36.4	18.6	6 colonies studied from 1 mouse injected
Same after 16 mos.	2	1.3	2.6	0.0	
Mouse injected with CH vaccine and SV-R culture	6	22.7	25.0	16.6	6 colonies studied from 1 mouse injected
Same after 16 mos.	2	0.0	0.0	0.0	
Mice injected with SV-R culture only	3	17.8	26.0	2.0	2 colonies studied from 1st mouse injected. 1 colony studied from 2nd mouse injected
Same after 16 mos.	2	1.05	1.1	1.0	
Stock SV culture	—	0.0	—	—	

*Only S colonies from hearts' blood cultures were studied.

influenced by the mode of reconversion. We especially wish to emphasize the fact that although the vaccine from the rabbit avirulent strain affords the necessary stimulus for reversion of R to S, it induces no change in the direction of a decreased virulence on the part of the reconverted form.

Of the rabbits injected with cultures representing the smooth forms of the R variant derived from the rabbit avirulent strain one animal

died after 4 days and from the heart's blood a pure culture of pneumococcus was recovered. We do not regard this result as demonstrating an increase in virulence on the part of the reconverted organisms since a further examination of the data will show that two animals, one of which was inoculated with the same culture and another with the organisms grown from the heart's blood of the animal which died, both recovered after exhibiting a bacteremia in all respects comparable

TABLE IV

Virulence for Rabbits of S Strains Reconverted from R

Rabbit No.	Origin of culture*	Amount of culture injected intravenously	Result	Remarks
		cc.	*hrs.*	
1	SV-R culture and CH vaccine	0.1	D 48	Heart's blood: Positive. Culture was the 4th daily transplant from original single colony fishing
2	SV-R culture and CH vaccine	0.1	D 96	Heart's blood: Positive. Same culture as used in rabbit 1 but preserved in defibrinated blood for 16 mos.
3	SV-R culture and SV vaccine	0.1	D 27	Heart's blood: Positive. Culture was the 4th daily transplant from single colony fishing
4	SV-R culture only	0.1	D 60 ±	Heart's blood: Positive. Culture had been preserved 16 mos. in defibrinated blood before testing
5	CH-R culture and SV vaccine	0.2	S	Culture obtained from original colony No. 10 fishing; preserved for 2 wks. in defibrinated blood
6	CH-R culture and SV vaccine	0.2	D 96	Heart's blood: Positive. Culture obtained from original colony No. 17 fishing; preserved for 2 wks. in defibrinated blood
7	CH-R culture and SV vaccine	0.2	S	Culture from original colony No. 17 fishing. Blood cultures at intervals gave curve typical of strain CH
8	CH-R culture and SV vaccine	0.2	S	Culture from heart's blood of rabbit 6 used for injection. Blood cultures at intervals gave curve typical of strain CH
9	CH-R culture only	0.2	S	Culture from original colony fishing; preserved for 2 wks. in defibrinated blood

* 17 hour blood broth cultures of S colonies obtained from cultures of heart's blood of mice injected with the combinations noted.

to that following the injection of strain CH. It is probable that the death of this animal was due to some other cause at a time when the low grade bacteremia characteristically produced by the avirulent form was still present. If, then, this explanation be correct, it may be stated that the R variant obtained from the rabbit avirulent strain and transformed again to the S under the influence of a vaccine prepared from the virulent strain does not exhibit any increased virulence but behaves in this characteristic like the rabbit avirulent strain from

which it originated. In addition, it may be noted from Table IV that this same R variant when injected in large quantity into mice produces an S form which is avirulent for the rabbit. Cultures of the CH-R variant reconverted in the presence of the homologous smooth vaccine were not tested for their rabbit virulence since there was no reason to believe that any difference from the parent strain would be observed.

SUMMARY AND DISCUSSION

The results which have been presented show that under the conditions of artificial cultivation at 37°C. definite differences exist between two smooth strains of Pneumococcus Type III both of which are highly virulent for mice by the intraperitoneal route, but which may be sharply distinguished in their virulence for rabbits. These differences consist in the size of the fully developed intact capsule and the interval of time required for its loss. The somewhat smaller capsule of the avirulent strain, well formed and easily demonstrable during the early period of growth, diminishes quickly, while the large capsule of the strain virulent for rabbits is retained for a considerably longer period. Closely correlated with the time at which this reduction of capsule occurs is the appearance of changes in the surface properties of the bacteria which are revealed by a shifting of the range of acid agglutination, susceptibility to clumping in anti-R serum and ingestion by normal adult human polymorphonuclear leucocytes and serum. Since it has been shown that these alterations as growth continues, result in a loss of characteristics which distinguish the strictly type specific, fully capsulated pneumococcus and ultimately lead to a state temporarily approximating that of the completely avirulent R form, and since under the experimental conditions they are inaugurated sooner, advance more rapidly and are more complete in the rabbit avirulent organism, we believe that they may partly account for difference in rabbit virulence of the two strains. In the following paper an attempt has therefore been made to correlate this behavior *in vitro* with the events attendant upon inoculation into the animal body.

The studies of Clark and Ruehl (16), Henrici (17), Bayne-Jones and Adolph (18) and others have demonstrated a marked increase in the size of the bacterial cell associated with the early phases of growth.

These authors have dealt chiefly with noncapsulated rod forms and even Clark and Ruehl who included cultures of various cocci do not make reference to variations in capsule size. Recently Seastone (19) has called attention to the large volume occupied by young capsulated streptococci. Similarly we have found that increase and decrease of Pneumococcus Type III volume appears to be due largely to the formation of capsule in young cultures and its subsequent loss as the organisms age. Because of the relatively great proportion of capsule in comparison with soma, a greater disparity exists between the volume of young and old pneumococci than that found by those who have studied bacteria lacking this structure. Of interest in connection with our observations are those of Preisz (20) on the nature of the capsules of virulent anthrax bacilli and strains attenuated by cultivation at 42.5°C. The latter produced soft, rapidly dissolving capsules while such structures in the former were characteristically firm and were retained by the bacilli for longer periods. This worker also noted in confirmation of the earlier work of others, that the capsules of *B. anthracis* are lost during the course of growth in serum media and in the subcutaneous tissues of the susceptible mouse.

We have demonstrated that the R variants derived under the same conditions from the two smooth strains of Pneumococcus Type III reveal certain characteristics by which they may be distinguished from each other in respect to cell and colony morphology, growth in broth, as well as growth at 41°C. (*cf.* Paper I). By employing the method of Griffith, these two R variants have been induced to revert to the S form. Following the injection into mice of the various possible combinations of living R variant and the killed S organisms of either rabbit virulent or avirulent strain, as well as very large numbers of the R variant alone, S forms emerged which in their various attributes, notably that of virulence for rabbits, resembled the original smooth strain from which the particular R variant involved was dissociated. The function of the smooth killed organisms in the process of transformation appeared to be only that of a stimulus toward reversion to the S. They apparently play no rôle in determining the virulence or the growth properties of the resulting S form.

These observations indicate that the factors involved in virulence are conditioned by stable physiological properties peculiar to the

individual strain and that although temporarily inactive during the R state, they are again resumed unaltered upon the transition to the S form. They serve also to reemphasize the fact, apparent from several studies but perhaps not sufficiently realized, that the R variants of the pneumococcus, even though obtained under the same conditions from the same type but from different strains, may vary definitely in their various attributes.

Finally, they strongly suggest that the degree of virulence of a given strain of a bacterial species may be determined not only by its ability to multiply in the environment of the host and to synthesize certain substances of definite chemical and antigenic properties, but also by the capacity to elaborate these in greater or lesser degree and under the conditions of parasitism within the animal body to maintain them in contact with the soma of the cell in such state that they afford an efficient barrier to the defensive mechanisms of the host.

CONCLUSIONS

1. Certain properties exhibited in culture by two smooth strains of Pneumococcus Type III, one virulent for rabbits and the other relatively avirulent for these animals, have been studied. No antigenic differences between these strains have been revealed by the cross-absorption of agglutinins from homologous antisera.

2. In young cultures the organisms of both strains possess well developed capsules. It has been shown, however, by stained preparations and measurements of relative volume, that the rabbit avirulent strain loses this capsule in dextrose serum broth cultures about 8 hours before it disappears in the case of the rabbit virulent organism. Similar results have been obtained when growth takes place in normal rabbit blood or serum. The rate at which loss of capsule occurs is independent of increased acidity attendant on growth.

3. Loss of capsule in both cases goes *pari passu* with marked shrinkage in volume, alterations in the zone of acid agglutination, susceptibility to agglutination in anti-R pneumococcus serum and to phagocytosis.

4. Because of these changes, older cultures of both strains appear to be composed largely of individuals which approximate the avirulent R form in respect to size and surface properties. In the rabbit avirulent

strain this approach to a state analogous to that of the R form begins earlier, proceeds more rapidly and goes further toward completion.

5. R variants dissociating from these two smooth strains of Pneumococcus Type III are distinguishable from one another in morphological and growth properties.

6. When reversion to the smooth type specific form of such R variants occurs, the organisms closely resemble in respect to the virulence for rabbits and other attributes the original strain from which the R variant was derived, regardless of whether killed organisms of the rabbit virulent or avirulent strains were employed in effecting the reversion.

7. On the basis of these findings it appears that the factors upon which the difference in virulence between two strains of smooth Type III pneumococcus may depend are the products of stable physiological processes of the bacterial cell which are retained during the transformation S→R→S.

BIBLIOGRAPHY

1. Enders, J. F., and Shaffer, M. F., *J. Exp. Med.*, 1936, **64**, 7.
2. Griffith, F., *J. Hyg.*, 1928, **27**, 113.
3. Dawson, M. H., *J. Exp. Med.*, 1930, **51**, 99.
4. Ward, H. K., and Lyons, C., *J. Exp. Med.*, 1935, **61**, 515.
5. Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, **26**, 537.
6. Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1933, **57**, 721.
7. Ward, H. K., and Enders, J. F., *J. Exp. Med.*, 1933, **57**, 527.
8. Enders, J. F., and Wu, C.-J., *J. Exp. Med.*, 1934, **60**, 127.
9. Gillespie, L. J., *J. Exp. Med.*, 1914, **19**, 28.
10. Hughes, T. P., *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 804.
11. Sherman, J. M., and Albus, W. R., *J. Bact.*, 1923, **8**, 127.
12. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42**, 727.
13. Hornus, G. J. P., and Enders, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 102.
14. Watson, E. L., and Cooper, G., *J. Exp. Med.*, 1930, **52**, 849.
15. Tillett, W. S., *J. Exp. Med.*, 1927, **45**, 713.
16. Clark, P. F., and Ruehl, W. H., *J. Bact.*, 1919, **4**, 615.
17. Henrici, A. T., Morphologic variation and the rate of growth of bacteria, Springfield, Illinois, C. C. Thomas, 1928.
18. Bayne-Jones, S., and Adolph, E. F., *J. Cell. and Comp. Physiol.*, 1932, **1**, 409.
19. Seastone, C. V., *J. Bact.*, 1934, **28**, 481.
20. Preisz, H., *Centr. Bakt., 1. Abt., Orig.*, 1911, **58**, 510.

EXPLANATION OF PLATE 20

The photographs were made from smears of dextrose serum broth cultures of Pneumococcus Type III, strains SV and CH, stained with the alcoholic methyl violet-basic fuchsin stain described in the text. Figures to the right of the black line are of strain CH, those to the left of strain SV.

FIGS. 1 and 2. Strain SV. 3 hour culture. Note completely developed capsule.

FIG. 3. Strain SV. 4 hour culture. Large intact capsule.

FIG. 4. Strain CH. 4 hour culture. Large intact capsule.

FIGS. 5 and 6. Strain SV. 6 hour culture. Capsules.

FIGS. 7, 8 and 10. Strain CH. 6 hour culture. Note diminished and imperfect capsule in the case of certain organisms and its absence in others.

FIG. 9. Strain SV. 10 hour culture. Large intact capsule. Compare with Fig. 15.

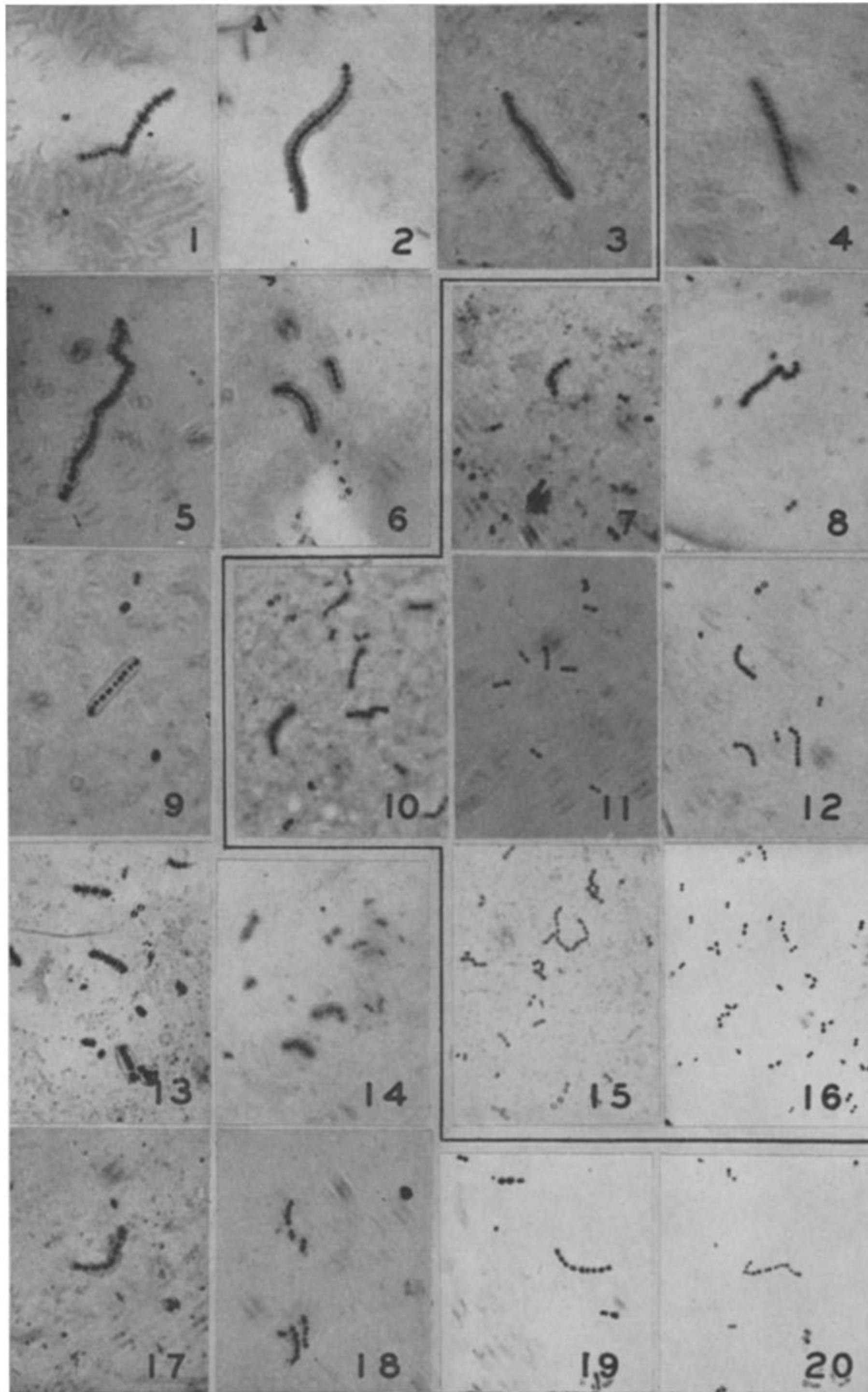
FIGS. 11 and 12. Strain CH. 7 hour culture. Note the almost complete absence of capsule.

FIGS. 13 and 14. Strain SV. 14 hour culture. Capsules are still present.

FIGS. 15 and 16. Strain CH. 10 hour and 14 hour cultures. Complete absence of capsules.

FIGS. 17 and 18. Strain SV. 16 hour culture showing in one instance large capsule, in others only traces of the structure.

FIGS. 19 and 20. Strain SV. 22 hour culture. Complete absence of capsules. Compare with Fig. 15.



(Shaffer *et al.*: Immunity to Pneumococcus Type III. II)