VARIATIONS IN THE PNEUMOCOCCUS INDUCED BY GROWTH IN IMMUNE SERUM.

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The change produced in bacteria by growth in specific immune sera has been studied for many years. Metchnikoff (1) in 1887 found that the virulence of anthrax bacillus seemed to be diminished by growth in anti-anthrax serum. Similar results were obtained by Charrin and Roger (2) and Roger (3) with *B. pyocyaneus*, pneumococcus, and streptococcus. Later investigations, however, showed that this apparent loss of virulence was due to the protective action of the immune serum present, since bacteria, freed from the serum in which they had been grown, showed no alteration in their virulence (Metchnikoff (4), Sanarelli (5), Issaeff (6)). The study of the effect of this treatment has been confined chiefly to observations on *B. choleræ*, *B. typhosus*, and other intestinal organisms (von Ransom and Kitashima (7), Müller (8), Eisenberg (9), Walker (10), Smith and Reagh (11), and Feiler (12)). Investigators have found that bacteria grown in homologous immune sera, or in the peritoneal cavities of immune animals, show marked increase in virulence and in resistance to bactericidal action, and a loss of agglutinability. The increase in virulence and in resistance to bactericidal action would seem to indicate a biological adaptation on the part of the bacteria, similar to the process of increased resistance which the animal organism develops when invaded by infectious agents. The loss of agglutinability of the organisms treated with immune serum has been explained as being possibly attributable to an inadequate receptor apparatus, this condition having been brought about by the injurious action of the immune serum upon the organism. Joos (13) has demonstrated that such an effect is brought about by heating typhoid bacilli. He finds that the agglutinin-producing substance, agglutinogen, of typhoid bacilli apparently consists of two elements, which he designates as α- and β-agglutinogen. The α-agglutinogen is easily destroyed by heating at 60–62°C., while the β-agglutinogen is heat-resistant. Injection of living unheated bacilli produces both α- and β-agglutinins, while the injection of heated bacilli produces only β-agglutinins. Cole (14) has found that inagglutinable typhoid strains possess less power to absorb agglutinins than do normal strains, and, when injected into animals, produce sera which are not as highly agglutinating, even for the injected strain, as the sera produced by highly agglutinable strains. These experiments seem to show that the receptor apparatus of the inagglutinable strains is not so complete as that of the agglutinable bacilli.

Recently Friel (15) has found that by growing pneumococci in immune serum they became agglutinable and phagocytable in normal rabbit serum, and less virulent for mice than the untreated strains.

The present investigation was undertaken in order to study the different types of pneumococcus with regard to this phenomenon and to determine whether any variation of type organisms occurs after treatment with Antipneumococcus Serum I or II.

The experiments were made with virulent strains of the pneumococcus, freshly isolated from the heart's blood of mice dead from pneumococcus septicemia, or obtained from blood cultures from cases of lobar pneumonia. In all experiments determinations of morphology, bile solubility, inulin fermentation, capsule formation, virulence, and agglutination were made before the strain was subjected to treatment. Cultures were then made in normal and immune serum bouillon, and, in some instances, as a control, in plain bouillon. All serum media were prepared by the addition of 0.5 cc. of serum to 4.5 cc. of the nutrient beef infusion bouillon used for routine culture medium, the immune serum bouillon containing one part in ten of a highly potent antipneumococcus horse serum, the normal serum bouillon containing the same amount of normal horse serum. Cultures were made by inoculation of 0.5 cc. of a bouillon culture of the strain to be tested, the tubes were then incubated at 37°C. for 18 hours, and after that time 0.5 cc. of this serum culture was reinoculated directly into another tube of serum bouillon. In some instances cultures were transferred daily, in others at weekly intervals, being kept in the refrigerator during the intervals between incubation. Two different series of subcultures of each strain tested were employed, one being cultured consecutively in immune serum bouillon, the other in normal serum bouillon. The manner of growth and morphology of the organisms were noted each time that a culture was transferred. It was noted that all strains grew luxuriantly under the conditions and could be kept growing, by repeated transfer, for indefinite lengths of time. Two strains have now been grown under such conditions for over a year. Type strains grown in the homologous immune serum showed at first complete agglutination, the growth sedimenting after 18 hours' incubation in a hard mass which could be broken apart only with difficulty. After five or six transfers of Type

I in homologous serum, the growth sedimented in a flocculent mass which could be easily shaken apart. In order to produce the same effect with Type II organisms, twelve to fifteen transfers in immune serum were necessary. The microscopic picture was that of the well known thread reaction, individual organisms presenting a swollen appearance and growing in long chains or clump formation. All strains retained the original characteristics of bile solubility, inulin fermentation, and reaction to Gram stain. Marked differences, however, between the immune and normal serum treated organisms were noted in growth on blood media, agglutination, capsule formation, pathogenicity for white mice and rabbits, and antigenic and opsonic reactions.

Type strains which had been grown for a number of transfers in immune serum lost the characteristic moist, confluent, greenish growth on blood media and grew in dry, isolated, brownish colonies which showed a tendency to produce hemolysis of the blood cells in the media. When grown in plain bouillon subsequent to serum treatment, these strains showed marked sedimentation, this characteristic persisting even after twenty-five passages in plain bouillon. They also showed less tendency to form methemoglobin than the normal serum treated strains.

Variations in Agglutination.

The change noted was the development of a non-specific agglutinative reaction by the immune serum treated strains. Those strains which previously were agglutinable only in the homologous serum, subsequently were agglutinated somewhat less completely in this serum, and were also agglutinated by heterologous immune sera, and in some instances even by normal serum. All agglutination tests were made according to the following routine. 0.5 cc. of the 1:10 serum culture was inoculated into 5 cc. of plain bouillon; after this subculture had grown from 4 to 6 hours, 0.5 cc. was inoculated into 25 cc. of plain bouillon, and this second subculture incubated for 18 to 20 hours. At the end of that time the bacteria were centrifugalized out, washed twice in saline, and an emulsion of these washed organisms in salt solution was used for the agglutination test. In this way, the error that would result from the pres-

ence of the immune serum in which the organisms had been grown was avoided. It will be seen that the last subculture contained serum in dilution of only 1:5,000; after washing twice in saline even this small amount must have been largely removed. Antipneumococcus serum does not agglutinate in a dilution of immune serum higher than 1:500, hence the reaction obtained could not have been due to a small amount of serum adhering to the bacteria. Agglutinations were made with the two types of antipneumococcus horse serum, and with normal horse serum. In each case one tube contained equal parts of the undiluted serum and bacterial emulsion, and the others 0.9 cc. of serum dilution and 0.1 cc. of bacterial emulsion. Tubes were kept in a water bath at 37°C. for 2 hours, and over night on ice, and readings were taken at the end of that time. Complete reaction (++) was recorded when the bacteria were entirely sedimented with marked precipitation, leaving the supernatant fluid clear; almost complete (+±), when the bacteria were sedimented with less marked precipitation; incomplete (+), when marked clumping could be seen but the clumps were still readily shaken from the bottom of the tube; partial (±), when the clumps had not sedimented, but could be distinguished macroscopically as a fine granulation; negative (−), when no clumping was present.

Table I shows the result of three agglutination tests, the organism in each instance having descended from the same mouse passage culture of Pneumococcus Type I. The original culture showed typical agglutination reaction, virulence, and protection by homologous serum. Subculture A was grown successively in Immune Serum I bouillon, Subculture B successively in normal serum bouillon, Subculture C successively in plain bouillon. After 4 immune serum treatments, Subculture A lost intensity of reaction with its homologous serum, from complete to incomplete, and became partially agglutinated with the heterologous serum. After 20 treatments in immune serum the reaction was still incomplete with the homologous serum and had become almost complete with the heterologous serum. After 15 transfers in plain bouillon, subsequent to 22 treatments in immune serum, the organisms had regained somewhat in intensity of reaction with the homologous serum, but still agglutinated partially with the heterologous serum. Subcultures B and C, on the contrary, maintained

TABLE I.

Agglutination Tests of Serum Treated Strain I 114, Pneumococcus Type I, 114 Animal Passages, before Serum Treatment.

Subcultures in each test were grown for two successive transfers in plain bouillon subsequent to serum treatment and a twice washed emulsion of centrifugalized organisms was used. Tube 1 in each case contained 0.3 cc. of bacterial emulsion and 0.3 cc. of undiluted serum; the remaining tubes contained 0.9 cc. of serum dilution and 0.1 cc. of bacterial emulsion. Readings were made after the tubes had been kept in a water bath at 37°C. for 2 hours and over night on ice.

Serum dilutions.	Subculture A treated in Immune Serum I.			Subculture B treated in normal serum.			Subculture C untreated.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
Strain I 114[7]*. 4 serum treatments. Nov. 6, 1915.									
Undiluted.	+	±	−	++	−	−	+	−	−
1:10	+	±	−	++	−	−	+	−	−
1:20	+	±	−	++	−	−	++	−	−
1:40	+	−	−	++	−	−	++	−	−
1:50	+	−	−	+	−	−	++	−	−
1:100	+			−			−		
1:200	±			−			−		
1:400	±			−			−		
1:500	−			−			−		
Salt control.	−			−			−		
Strain I 114[23]. 20 serum treatments. Nov. 30, 1915.									
Undiluted.	+	+±	−	++	−	−	++	−	−
1:10	+	+±	−	++	−	−	++	−	−
1:20	+	+±	−	++	−	−	++	−	−
1:40	+	+±	−	++	−	−	++	−	−
1:50	+	+±	−	++	−	−	++	−	−
1:100	+			−					
1:200	+			−					
1:400	+			−					
1:500	+			−					
Salt control.	−			−					

TABLE I—*Concluded.*

Serum dilutions.	Subculture A treated in Immune Serum I.			Subculture B treated in normal serum.			Subculture C untreated.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
Strain I 114[38]. 15 transfers in plain bouillon subsequent to serum treatment. Dec. 21, 1915.									
Undiluted.	±	±	−	+	−	−			
1:10	++	±	−	+	−	−			
1:20	+	±	−	+	−	−			
1:40	+	±	−	+	−	−			
1:50	+	±	−	+	−	−			
1:100	+			+					
1:200	+			−					
1:400	−			−					
1:500	−			−					
Salt control.	−			−					

* In all the tables the numerals after the culture indicate the number of mouse passages. The exponent indicates the number of generations removed from the last passage.

throughout the course of treatment the same intensity of reaction with the homologous serum and did not become agglutinated either in the heterologous or in the normal serum.

Table II shows the result of three agglutination tests made with two subcultures, both from the same mouse passage culture of a Pneumococcus Type II, which showed typical agglutination reaction, virulence, and protection before treatment. Subculture A was grown successively in Immune Serum II bouillon, Subculture B successively in normal serum bouillon. After 53 treatments with immune serum, Subculture A was only partially agglutinated in the homologous Serum II, but was completely agglutinated in the heterologous Serum I, and incompletely agglutinated in normal serum. After 75 treatments in immune serum, it was still only partially agglutinated in the homologous serum, completely agglutinated in the heterologous, and, this time, not agglutinated in normal serum. After 47 transfers in plain bouillon subsequent to serum treatment, the partial agglutination with the homologous serum and complete agglutination with the

TABLE II.

Agglutination Tests of Serum Treated Strain II 34, Pneumococcus Type II, 34 Animal Passages, before Serum Treatment.

The same technique was used as in the tests in Table I.

Serum dilutions.	Subculture A treated in Immune Serum II.			Subculture B treated in normal serum.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
Strain II 34^{68}. 53 serum treatments.						
Oct. 1, 1915.						
Undiluted.	++	±	+	−	++	−
1:10	++	±	+	−	++	−
1:20	++	±	+	−	++	−
1:40	+	±	+	−	++	−
1:50	+	±	+	−	++	−
1:100		±			++	
1:200		±			−	
1:400		−			−	
1:500		−			−	
Salt control.		−			−	
Strain II 34^{90}. 75 serum treatments.						
Dec. 2, 1915.						
Undiluted.	++	±	−	−	++	−
1:10	++	±	−	−	++	−
1:20	++	±	−	−	++	−
1:40	++	±	−	−	++	−
1:50	++	±	−	−	±	−
1:100	++	±			−	
1:200	−	−			−	
1 400		−			−	
1:500		−			−	
Salt control.		−			−	
Strain II 34^{116}. 47 transfers in plain bouillon subsequent to serum treatment.						
Dec. 2, 1915.						
Undiluted.	++	±	−	−	++	−
1:10	++	±	−	−	++	−
1:20	++	±	−	−	++	−
1:40	+	±	−	−	++	−
1:50	+	±	−	−	++	−
1:100		±			−	
1:200		−			−	
1:400		−			−	
1:500		−			−	
Salt control.		−			−	

heterologous serum persisted. In this experiment, also, the organisms grown in normal serum were still completely agglutinated with homologous serum and were in no instance agglutinated with the heterologous or normal serum. It is to be noted, however, in spite of the incompleteness of the agglutination of the treated organisms in the homologous serum, that the agglutination titer in homologous serum was higher than that in heterologous serum, and also higher than that of normal untreated strains in homologous serum.

These tests were repeated a number of times with many different strains, always with the same result; the longer a strain was treated with its homologous serum, the less agglutinable it became in that serum, and the more agglutinable in the heterologous serum. On the contrary, the control strains cultured in normal serum and plain bouillon, retained throughout the experiments the typical agglutination with the homologous serum and were never agglutinated by the heterologous or normal serum. It was also noted that the immune serum treated strains sometimes were agglutinated with normal horse serum, but they did not agglutinate spontaneously in salt solution, nor did they agglutinate in other immune sera, such as diphtheria and tetanus antitoxin, anti-influenza serum, nor in an immune sheep serum obtained by the injection of an antigen prepared by the solution of pneumococci in sodium cholate. Bull (16) has tested an immune serum treated strain of Type I and found that it is agglutinated *in vivo* and *in vitro* by normal rabbit serum, while the control strain is not.

Variations in Virulence.

Simultaneous with the change in agglutinability, the immune serum treated cultures showed a pronounced loss of virulence for white mice and rabbits. It often required a million times more of an immune serum treated culture to kill than of the control normal serum treated culture. Virulence tests were always made with cultures at least twice removed from the serum treated culture in which 1 cc. contained only $\frac{1}{5,000}$ part of immune serum. As 0.1 cc. of this serum is required to protect against 0.1 cc. of a normal strain, this loss of virulence cannot be attributed to the protective value of the serum present. In rabbits it was found

that the washed bacteria from 15 cc. of a bouillon culture of treated organisms would not kill, while 0.000005 cc. of culture of untreated organisms killed in 24 hours. In white mice it often required the bacteria from 5 cc. of a culture of treated organisms to kill, while 0.000001 cc. of culture of untreated organisms killed in 24 hours.

Table III shows the comparative virulence of the immune and normal serum treated cultures of Pneumococcus Type I and Pneumococcus Type II, the agglutination tests of which are given in Tables I and II. The virulence of Subculture I 114 B, treated 12 times with normal serum was such that 0.000001 cc. of a 20 hour bouillon culture killed in 56 hours, while the fatal dose of Subculture A, treated 12 times with Immune Serum I, was 0.5 cc. of a 20 hour bouillon culture. After 20 treatments with immune serum, 1 cc. of a broth culture of Subculture A did not kill, and after 46 treatments the

TABLE III.

Virulence of Serum Treated Strains of Pneumococci.

The tests were made by intraperitoneal injection into white mice. All serum cultures were transferred at least twice before injection into such amounts of bouillon that the dilution of serum in the culture used was at least 1:5,000. Autopsies were performed on all animals and smears were made from the peritoneal exudate. Pneumococci were found in all cases.

Test I.

Virulence, Pneumococcus Type I.

Amount of culture injected.	12 serum treatments.		20 serum treatments.		46 serum treatments.	
	Treated in Immune Serum I.	Treated in normal serum.	Treated in Immune Serum I.	Treated in normal serum.	Treated in Immune Serum I.	Treated in normal serum.
cc.						
5.0	—	—	—	—	D. 18 hrs.	—
1.0	—	—	S.*	—	S.	—
0.5	D. 24 hrs.	—	S.	—	S.	—
0.1	S.	—	S.	—	—	—
0.01	S.	—	S.	—	—	—
0.001	S.	—	S.	—	—	—
0.0001	S.	D. 18 hrs.	S.	D. 26 hrs.	—	D. 36 hrs.
0.00001	D. 24 hrs.†	" 56 "	—	" 25 "	—	" 36 "
0.000001	—	" 56 "	—	" 25 "	—	" 36 "

TABLE III—*Concluded.*

Test II.

Virulence Tests of Serum Treated Pneumococcus Type II Made with Cultures Which Had Received 27 and 61 Transfers in Plain Bouillon Subsequent to 55 Serum Treatments.

Virulence, Pneumococcus Type II.

Amount of culture injected.	27 transfers in plain bouillon.		61 transfers in plain bouillon.	
	Treated in Immune Serum II.	Treated in normal serum.	Treated in Immune Serum II.	Treated in normal serum.
cc.				
2.0	D. 18 hrs.	—	—	—
1.5	—	—	S.	—
1.0	S.	—	S.	—
0.5	D. 36 hrs.	D. 18 hrs.	S.	—
0.1	S.	" 18 "	S.	—
0.01	S.	" 36 "	—	—
0.001	S.	" 24 "	—	—
0.0001	—	" 24 "	—	D. 23 hrs.
0.00001	—	" 20 "	—	" 48 "
0.000001	—	—	—	" 48 "

* In the tables D. stands for died; S., for survived. The figures represent the number of hours after injection before the death of the animal.

† No pneumococci were found in the heart's blood of this animal.

animal receiving the bacteria from 1 cc. of a broth culture of Subculture A survived. Subculture B, however, which was treated an equal number of times with normal serum, maintained a high virulence, 0.000001 cc. of a broth culture killing regularly.

In Test II, Table III, is shown the comparative virulence of an immune and normal serum treated culture of Pneumococcus Type II 34. Each culture had received 55 serum treatments and had been passed subsequently through a large series of broth transfers. After 27 transfers in plain broth the fatal dose of the immune serum treated culture was 0.5 cc., or more, while 0.00001 cc. of the normal serum treated strain killed a mouse in 20 hours. After 61 transfers in plain bouillon 1.5 cc. of the immune serum treated culture failed to cause death, although 0.000001 cc. of the normal serum treated culture was fatal to a mouse in 48 hours.

It will be seen from these tables that the longer the pneumococcus is grown in immune serum the less virulent it becomes, while the same strain grown in normal serum retains its original virulence even after long periods of growth in plain bouillon. The immune serum treated organisms retain the avirulent characteristics after as many as 75 transfers in plain bouillon subsequent to serum treatment. This fact demonstrates conclusively that the loss of virulence is not due to the protective action of the immune serum present.

A study of the capsule formation in the immune and normal serum treated cultures demonstrated the interesting fact that while the latter upon injection into mice formed capsules easily demonstrable by the Hiss method, the immune serum treated organisms showed no demonstrable capsules under similar conditions. It is possible that the loss of virulence noted may in some way be related to the apparent absence of capsule formation.

Variations in Phagocytability.

The phagocytability of the organisms grown in immune serum and of those grown in normal serum was tested with guinea pig leukocytes in the presence of normal and immune horse serum by the Neufeld method. The organisms grown in normal serum were phagocyted only in the presence of immune serum. Those grown in immune serum after at least two subsequent broth passages were phagocyted in the presence of both immune and normal horse serum. The organisms grown in immune serum were also phagocyted *in vivo* in the normal rabbit, a reaction which does not take place with the normal virulent pneumococcus. Growth in immune serum has, therefore, made susceptible to the phagocytic action of guinea pig leukocytes a culture of pneumococcus previously resistant to such action. This phenomenon may also be related to the loss of virulence which occurs as the result of growth in immune serum, since it is known that non-virulent pneumococci are more readily phagocyted than highly virulent strains.

Variations in Antigenic Properties.

In order to test the possibility of variation in the antigenic properties of immune serum treated strains, two antigens were prepared in the following manner. Emulsions of washed bacteria, killed by heating at 56°C. for 45 minutes, were prepared from equal amounts of two bouillon cultures of Pneumococcus Type II 34, one of which had been treated for 60 successive transfers with Serum II, the other for the same number of transfers with normal horse serum. Immune sera were prepared from these antigens by intravenous injection of rabbits. The immunization of each animal was carried out in a corresponding manner, and the serum obtained for the tests at comparable intervals of time. The immune rabbit sera thus prepared were tested for their agglutination reaction with normal and immune serum treated pneumococci of both Types I and II.

Examination of Table IV shows that the immune response as measured by agglutinins is slower in the rabbits immunized with immune serum treated pneumococci than in those immunized with the normal serum treated organisms. It is further evident that the serum of rabbits immunized to immune serum treated Pneumococcus Type II contains agglutinins for the immune serum treated organisms of both Types I and II, but does not possess antibodies for the strains treated

TABLE IV.

Agglutination Reaction of Sera from Rabbits Immunized to Normal and Immune Serum Treated Strains of Pneumococcus Type II.

Agglutination Reaction of Sera Obtained 4 Weeks after the Beginning of Immunization.

Culture used for agglutination.	Serum from rabbits immunized with immune serum treated Pneumococcus Type II.		Serum from rabbits immunized with normal serum treated Pneumococcus Type II.	
	Rabbit 29C	Rabbit 30C	Rabbit 31C	Rabbit 32C
Normal Pneumococcus Type II	−	−	++	++
Immune serum treated Pneumococcus Type II	−	−	±	±

TABLE IV—*Concluded.*

Agglutination Reaction of Sera Obtained 6 Weeks after the Beginning of Immunization, Including Cross Agglutination Reactions with Normal and Immune Serum Treated Pneumococci Types I and II.

Dilutions of serum.	Serum from rabbit immunized with immune serum treated Pneumococcus Type II.				Serum from rabbit immunized with normal serum treated Pneumococcus Type II.			
	Normal Serum Treated Culture II.	Immune Serum Treated Culture II.	Normal Serum Treated Culture I.	Immune Serum Treated Culture I.	Normal Serum Treated Culture II.	Immune Serum Treated Culture II.	Normal Serum Treated Culture I.	Immune Serum Treated Culture I.
Undiluted.	−	+	−	+	++	+	−	+
1:10	−	+	−	+	++	+	−	+
1:20	−	+	−	+	++	+	−	+
1:40	−	+			+	+		
1:50		+			+	+		
1:100		+			+	+		
1:200		+			−	+		
1:400		−			−	−		

with normal serum. On the other hand, the sera of rabbits immunized to a Type II pneumococcus which had been grown in normal serum for a similar number of transfers, agglutinated not only the homologous normal strain, but reacted, although less sharply, with immune serum treated pneumococci of both Types I and II.

Variations in Absorption Properties.

It has been demonstrated by Avery (17) that absorption of Antipneumococcus Serum I or II with the homologous pneumococcus removes from the serum all agglutinins for the homologous organism, while saturation of Serum I with a Type II organism does not remove the agglutinins present for Type I, and the same result is obtained when an attempt is made to exhaust the antibodies of Serum II with Pneumococcus Type I. Saturation of the Antipneumococcus Serum I or II with homologous immune serum treated organisms, removes all agglutinins for both homologous and heterologous immune serum treated pneumococci, but does not remove the agglutinins for the homologous normal serum treated organisms. It is also found that saturation of antipneumococcus serum with

the homologous type of normal serum treated organisms removes the agglutinins not only for these pneumococci, but for all immune serum treated strains.

Comparative Absorption of Antipneumococcus Serum with Immune Serum Treated and Normal Serum Treated Pneumococci.

Technique.—To 5 cc. of Antipneumococcus Serum II was added the washed bacterial residue from 25 cc. of a 20 hour bouillon culture of Pneumococcus Type II which had been treated for 65 transfers with Immune Serum II. The mixture was kept at 37°C. for 30 minutes and for 18 hours at ice temperature. A similar mixture was prepared by adding to the same amount of Serum II the bacterial residue from 25 cc. of a 20 hour bouillon culture of a strain of Pneumococcus Type II which had been treated for 65 transfers with normal horse serum, and this mixture was also kept at 37°C. for 30 minutes and for 18 hours at ice temperature. At the end of that time both mixtures were centrifugalized and the clear supernatant fluids again absorbed with fresh bacterial residues of the immune and normal serum treated cultures. Agglutination tests were then made with these absorbed sera.

TABLE V.

Agglutination of Antipneumococcus Serum II before and after Absorption with Normal and Immune Serum Treated Strains of Pneumococcus Type II.

Strains used for agglutination.	Before absorption.	After absorption with normal serum treated Type II.	After absorption with immune serum treated Type II.
Pneumococcus Type II, normal serum treated	++	−	++
Pneumococcus Type II, immune serum treated	++	−	−
Pneumococcus Type I, normal serum treated	−	−	−
Pneumococcus Type I, immune serum treated	+	−	−

Table V gives the result of an absorption experiment with Antipneumococcus Serum II. Absorption was repeated until the normal serum treated organisms had absorbed from the serum all the agglutinins for this strain. This serum then had lost, also, the agglu-

tinins for the immune serum treated organisms which it formerly contained, while the serum which had been absorbed with equal amounts of the immune serum treated organism, although it had lost the agglutinins for the homologous immune serum treated pneumococcus, still retained an apparently undiminished quantity for the homologous normal serum treated organism.

Reversion to Type of the Altered Strains on Animal Passage.

Attempts were made to ascertain whether these variations in agglutinability, virulence, and capsule formation would persist after repeated animal passage. This study revealed the fact that all strains so far tested reverted to the original type by passage through the animal body, the number of passages required for this reversion depending upon the number of treatments in immune serum which the organism had received. Strains which had received from 6 to 12 treatments regained virulence and specific agglutinability upon 1 animal passage, while it required 3 or more successive passages to restore the virulence and agglutinability of strains which had received from 50 to 100 serum treatments.

TABLE VI.

Effect of Animal Passage upon Agglutination and Virulence of Serum Treated Pneumococci.

Test I.

Agglutination and Virulence Tests Made before and after Animal Passage of Culture I 112[9], Which Had Received 6 Serum Treatments and 2 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after animal passage.	Result before animal passage.	Result after animal passage.
	Virulence of organisms treated in Immune Serum I.		Virulence of organisms treated in normal serum.	
cc.				
1.0	D. 16 hrs.	D. 18 hrs.	D. 16 hrs.	—
0.2	" 40 "	" 18 "	" 16 "	—
0.1	" 40 "	" 18 "	" 16 "	D. 18 hrs.
0.01	S.	" 18 "	" 20 "	" 18 "
0.001	S.	" 40 "	" 24 "	" 20 "
0.0001	S.	" 22 "	" 40 "	" 20 "

TABLE VI—*Continued.*

Agglutination of Culture I 112[9], before and after Animal Passage.

Serum used for agglutination.	Culture treated in Immune Serum I.		Culture treated in normal serum.	
	Result before animal passage.	Result after animal passage.	Result before animal passage.	Result after animal passage.
Immune Serum I	+	++	++	++
" " II	±	−	−	−
Normal horse serum	++	−	−	−

Test II.

Agglutination and Virulence Tests Made before and after Successive Animal Passages of Culture I 114[62], Which Had Received 59 Serum Treatments and Subsequently 2 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after first animal passage.	Result after second animal passage.
Virulence of organisms treated in Immune Serum I.			
Bacteria from 10 cc. culture	D. 16 hrs.	−	
" " 5 " "	" 16 "	−	
2.0 cc. culture	" 16 "	D. 16 hrs.	D. 36 hrs.
1.5 " "	S.	S.	−
1.0 " "	S.	S.	S.
0.5 " "	S.	−	−
0.1 " "	−	S.	−
0.001 " "	−	−	S.
Agglutination in Serum I	+	+	++
" " " II	±	±	−
" " normal serum	±	+	−
Virulence of organisms treated in normal serum.			
0.01 cc. culture	D. 36 hrs.	−	
0.001 " "	" 20 "	−	
0.00001 " "	" 25 "	D. 22 hrs.	
0.000001 " "	" 36 "	" 21 "	
Agglutination in Serum I	++	++	
" " " II	−	−	
" " normal serum	−	−	

TABLE VI—*Concluded.*

Test III.

Agglutination and Virulence Tests Made before and after Successive Animal Passages of Culture II 34[124], Which Had Received 109 Serum Treatments and Subsequently 4 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after first animal passage.	Result after second animal passage.	Result after third animal passage.
Organisms treated in Immune Serum II.				
Bacteria from 10 cc. culture....	D. 36 hrs.	–	–	–
" " 5 " "	S.	–	–	–
2.0 cc. culture	–	D. 18 hrs.	–	–
1.0 " "	S.	" 18 "	–	–
0.5 " "	–	" 18 "	–	–
0.1 " "	–	" 18 "	D. 16 hrs.	–
0.001 " "	–	" 18 "	–	D. 36 hrs.
0.0001 " "	–	" 36 "	D. 16 hrs.	" 36 "
0.00001 " "	–	–	" 20 "	" 36 "
0.000001 " "	–	–	" 24 "	" 36 "
Agglutination in Serum I.....	+	+	+	–
" " " II.....	++	++	++	++
" " normal serum.	+	–	–	–
Organisms treated in normal serum.				
0.01 cc. culture.....	D. 16 hrs.	–		
0.001 " "	" 16 "	–		
0.00001 " "	" 20 "	D. 24 hrs.		
0.000001 " "	" 22 "	" 36 "		
Agglutination in Serum I.....	–	–		
" " " II.....	++	++		
" " normal serum.	–	–		

Table VI shows the result of animal passage of three different strains of immune and normal serum treated pneumococci. Test I shows the result of 1 animal passage of a Pneumococcus Type I which had received 6 serum treatments, original virulence and typical agglutinability being regained upon 1 animal passage. Before passage it required 0.1 cc. of culture of the immune serum treated organism to kill mice in 40 hours, and the organisms were agglutinated with Serum II and normal serum; after passage 0.0001 cc. of culture killed

in 22 hours and the bacteria were agglutinated only in Serum I. Test II shows the result of 3 animal passages of a Pneumococcus Type I which had received 59 serum treatments. In this case it required 3 animal passages to cause the strain to revert to original agglutinability, and the virulence had not been materially raised by this number of passages. Test III shows the result of 4 animal passages of a Pneumococcus Type II which had received 109 serum treatments. With this strain 4 passages were required to restore the original agglutinability, although the virulence was raised by 1 passage. In all cases control tests were made with a normal serum treated organism, which had neither lost in virulence nor in specific agglutinability. A great number of similar tests have demonstrated that these immune serum treated strains are readily caused to revert to the original type by animal passage.

DISCUSSION.

The effect of the treatment of pneumococci with immune serum may be attributed to a suppression of certain receptors, similar to the phenomenon observed by Cole with inagglutinable typhoid strains, since the serum treated pneumococci become less specifically agglutinable, can no longer absorb the agglutinins for the normal strains, and, when injected into the animal body, do not produce agglutinating sera for the normal strains.

The effect produced on the virulence of the pneumococcus by immune serum treatment is not identical with that produced in other bacteria by treatment with immune sera. The pneumococcus, instead of becoming more virulent, (serum-fast or immune) with immune serum treatment, becomes much less virulent. This loss of virulence is one of the most noticeable effects of the treatment with the immune serum and, as mentioned before, cannot be explained by the protective action of the serum present, since it persists after 60 or 70 transfers in plain bouillon subsequent to serum treatment, when the amount of serum present in the culture is too small to compute, and after the organisms have passed through so many generations that there could surely be no immune substances from the serum still adherent to them. It would seem that some biologic change must have

taken place in the organism, transforming it from the virulent to the avirulent type. This inference is confirmed by the fact that these strains rendered avirulent have lost capsules and are phagocyted in normal serum.

At present we have no explanation to offer in regard to this difference between the virulence of the typhoid bacillus and the pneumococcus when grown in their immune sera. Since antityphoid serum is bactericidal and reacts best in the presence of complement, and since antipneumococcus serum possesses no demonstrable bactericidal properties and its agglutinating and protective action is not increased by the addition of complement, it is possible that the different results of serum treatment upon these bacteria with such widely separated biologic characteristics may, in some way, be attributable to the differences in the properties of these two sera.

It is interesting to note that the variations in pneumococci produced by treatment with immune serum do not persist after animal passage. Very few passages through the animal body cause the changed organism to revert to the original type with characteristic agglutinability, virulence, etc.

CONCLUSIONS.

1. The growth of virulent pneumococci in homologous immune serum produces (*a*) variations in agglutinability, (*b*) decrease in virulence, (*c*) inhibition of capsule formation, (*d*) increase in phagocytability with normal serum, and (*e*) change in absorption power and antigenic properties.

2. Reversion to the original type in these changed forms takes place upon animal passage.

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