STUDIES ON NATURAL IMMUNITY TO PNEUMOCOCCUS TYPE III

III. Correlation of the Behavior in Vivo of Pneumococci Type III Varying in Their Virulence for Rabbits with Certain Differences Observed in Vitro

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Plate 21

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In an accompanying paper it has been shown that two strains of Pneumococcus Type III differing in rabbit virulence could be distinguished in respect to the rate at which the capsule diminished during cultivation in various media. Concomitantly with the progressive loss of capsular substance, changes in the surface properties of the organisms were demonstrated which ultimately, but after different periods, reduced each strain to a state in many respects resembling that of the R form. The principal objective of the experiments to be presented here was to reveal a relationship between these facts and the events which ensue when the organisms are introduced into the animal body.

Materials and Technique

Strains of Pneumococci.—The two strains of Pneumococcus Type III designated SV and CH, and the R variant derived from each as described in Paper II were used throughout these experiments, as well as a stock R derivative from Pneumococcus Type I.

Animals.—In all cases, unless specific descriptions are given in the text, albino rabbits ranging in weight from 1600 to 2000 gm. and white mice of 15 to 20 gm. weight were used.

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Media.—Cultures of all the strains of pneumococci were regularly grown in the rabbit serum dextrose infusion broth medium described in Paper II, except in certain cases noted in the text. Seed cultures and stock cultures were grown in the same media as previously described.

Blood Cultures.—Blood was removed from the ear vein of rabbits into a tuberculin syringe and measured portions of serial dilutions plated in Petri dishes containing 0.5 cc. of defibrinated horse blood and melted infusion agar.

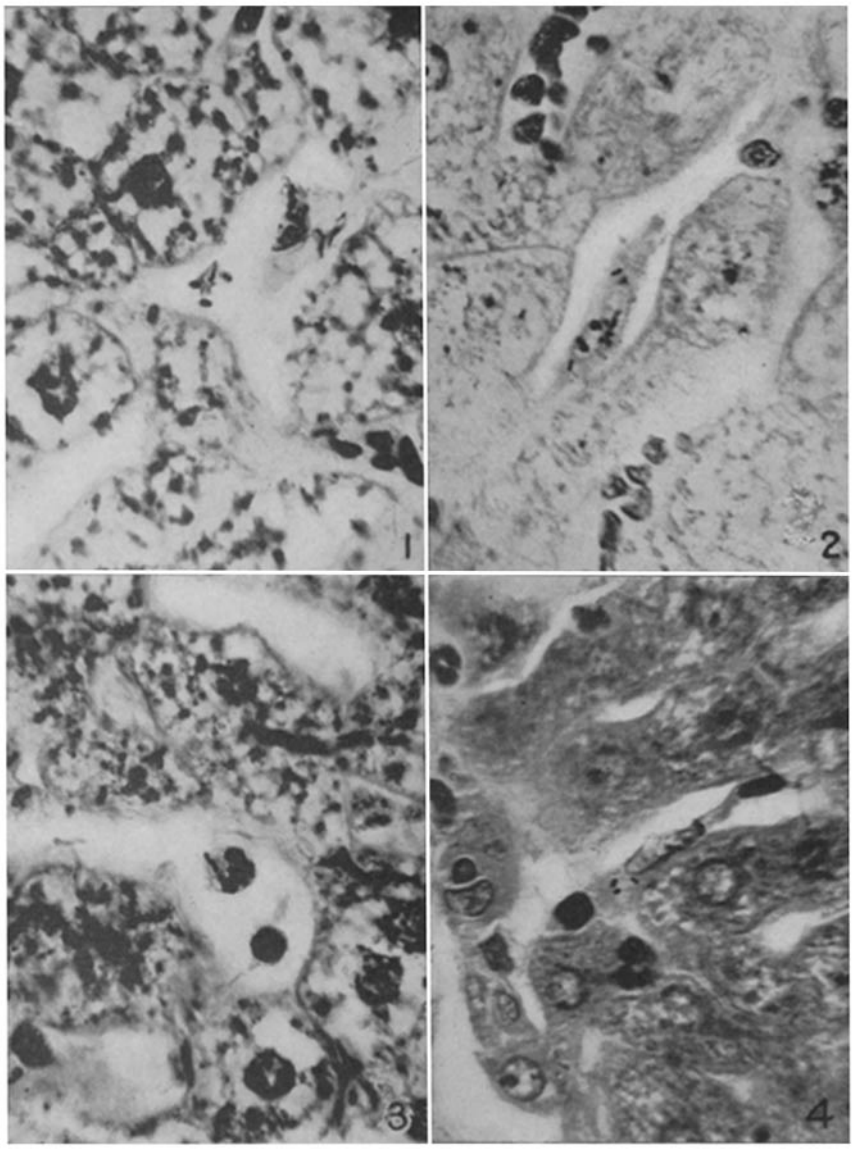
Phagocytosis.—

1. In Vitro.—In examining the phagocytic properties of rabbit leucocytes mixed with rabbit defibrinated blood, the method of obtaining and standardizing the materials was that of Robertson and Sia (1) as modified by Enders and Wu (2) for use in bactericidal measurements, with the exception that leucocytic exudate was produced within the peritoneum by means of large injections of saline, instead of in the pleural cavity. The cells and serum of mice were obtained in the following manner: 24 hours before the experiment, normal mice were injected intraperitoneally with 1.0 cc. of physiological salt solution and again with the same quantity after 22 hours. 2 hours later, the animals were anesthetized with ether and bled from the heart, using a tuberculin syringe and a 27 gauge needle. The blood, usually amounting to about 1 cc., was defibrinated in a test tube with a few glass beads. The peritoneum was then washed out with a small quantity of 1.5 per cent citrate solution. After twice washing the cells thus obtained in saline, the defibrinated blood from the same animal was added to them. They were then gently mixed and used in the same way as that of the similar combination of rabbit cells and serum.

2. In Vivo.—An exudate of leucocytes was induced in the pleural cavity of rabbits by the injection of 4 cc. of sterile infusion broth. 20 hours later, 0.2 cc. of thick suspension of the organisms centrifuged from 10 cc. of 23 hour broth cultures was injected. At intervals thereafter, samples of exudate were aspirated into a tuberculin syringe. Smears of the exudate were stained with Wright and Gram stains. For the latter, the preparation was fixed for 1 to 2 minutes in methyl alcohol. 2 per cent aqueous neutral red was employed as a counterstain. Measured volumes of exudates were diluted, and plates poured according to the procedure described for blood cultures.

For observing the phagocytosis within the peritoneal cavities of mice, 0.5 cc. of sterile saline was administered twice intraperitoneally into a series of mice at 16 to 18 hour intervals. 4 hours later, 0.1 cc. of organisms resuspended in sterile broth after centrifugation of cultures of various ages was injected. At subsequent intervals, a mouse was sacrificed and smears made from the peritoneal exudate.

In studying the phagocytosis accomplished by the fixed tissue cells of the rabbit, the bacteria from 20 to 80 cc. of blood broth cultures of pneumococci were injected in 1 to 2 cc. of sterile broth. After the sacrifice of the animals at varying periods subsequent to infection, portions of various organs were fixed in Zenker's solution. From these sections were prepared and stained with Gram-Weigert and Giemsa stains.



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

Technique Employed in Estimating Number of Viable Organisms in Various Tissues.—At intervals after intravenous infection of rabbits, portions of the various organs were removed and their weight determined to the nearest milligram, then ground for about 10 minutes in a mortar containing sterile sand. To the tissue sufficient sterile broth was added to give a 1/50 suspension, from which serial dilutions were then prepared, and 0.1 cc. portions plated according to the method used for culturing the blood.

EXPERIMENTAL

The Correlation of Capsule with the Initial Clearing of the Organisms from the Blood Stream

Following the intravenous inoculation of 12 to 14 hour broth cultures into rabbitts, Tillett (3) has demonstrated a difference in the behavior of rabbit virulent and avirulent strains of Pneumococcus Type III. The organisms of the former strain either remained in the blood stream in large numbers or underwent a certain degree of initial removal. After a stationary period, they began to increase steadily, followed by the death of the animal. On the other hand, with the avirulent strains the numbers in the blood rapidly underwent a marked reduction with a subsequent moderate increase after several hours. Thereafter, a low fluctuating degree of bacteremia was maintained for 4 or 5 days, followed by recovery. In the experiments which follow, we have employed the same method, except that more exact estimates of the numbers of organisms present in the blood were obtained in studying the rate at which removal of the rabbit virulent strain SV and the rabbit avirulent strain CH took place when cultures of various ages were injected. The results, when broth cultures of the age (12 to 14 hours) employed by Tillett were injected, agree in general with his. When, however, still older broth cultures, or young, encapsulated organisms or "animal" bacteria were introduced into the blood stream, a very different type of initial elimination curve was secured. In Text-figs. 1 and 2 are presented two series of curves obtained by plotting the logarithms of the number of organisms of strains CH and SV per cubic centimeter of blood at different intervals during the period immediately subsequent to infection. Each curve represents the course of the bacteremia in a rabbit inoculated with one of the following: a culture of varying age, mouse peritoneal washings or the blood of an infected rabbit taken shortly before death. The experimental data used in drawing the curves, as well as additional material, are presented in Tables I and II. It will be seen that the organisms derived from the animal body (mouse peritoneum or blood from an infected rabbit) or from very young (3 hour broth) cultures of strain CH remain in large numbers in the blood during the first 4 hours of experiment. The aging of the CH culture during artificial cultivation, however, very rapidly induces a change in the organism which is reflected in the increased rate of removal from the blood. Thus, the numbers of a 4½ hour broth culture are reduced about one hundredfold within 40 minutes. After 5 hours growth in broth, the organisms have become so altered that at the

end of 30 minutes only about 1/10,000 of those present after 1 minute are found in the blood. 10 hour broth cultures of this strain disappear so swiftly that after 1 hour only 10 organisms or less per cc. of blood are found.

In general, the curves obtained with comparable cultures of strain SV follow the same spatial pattern. The majority of animal organisms

TAB

Intravenous Inoculation of Norı
Results of Blood Cultures (Expressed as Numbers of Viab

Time interval elapsing between infection and blood culture	Organisms from mouse peritoneum				Organi of inf
	Rabbit 8 (Text-fig. 1, curve A)	Rabbit 10	Rabbit 21	Rabbit 17	Rabbi with blo (Text-f
min.					
1	3.6×10^7	3.9×10^7	2.9×10^8	1.5×10^8	$6.1 \times$
10	2.5×10^7	3.0×10^7	—	1.7×10^8	$2.2 \times$
20	3.4×10^7	1.4×10^7	4.7×10^7	1.3×10^8	
30–40	1.1×10^7 (40 min.)	7.3×10^6 (40 min.)	3.2×10^7 (40 min.)	1.6×10^8 (40 min.)	$3.5 \times$
hrs.					
1	1.3×10^7	2.6×10^6	2.3×10^7	3.5×10^7	$1.4 \times$
2	6.7×10^6	8.3×10^3	5.8×10^5	1.2×10^8	$4.2 \times$
3	—	—	1.6×10^4	5.6×10^7	$1.3 \times$
4	1.9×10^5	2.0×10^3	2.9×10^5	—	$1.2 \times$
5	—	—	5.0×10^4	—	
16–20	2.5×10^7 (18 hrs.)	—	—	2.0×10^8 (19 hrs.)	$2.3 \times$
21–25	—	2.0×10^3 (23 hrs.)	3.0×10^5 (22 hrs.)	2.9×10^8 (22 hrs.)	$1.1 \times$
26–40	—	—	9.2×10^3 (26 hrs.) 1.4×10^3 (28 hrs.)	—	
41–52	—	200 (46 hrs.)	2.2×10^3 (47 hrs.) 100 (52 hrs.)	—	
69–72	—	23 (72 hrs.)	0 (72 hrs.)	—	2 (69 h
92–97	—	0 (97 hrs.)	0 (96 hrs.)	—	
116–140	—	—	—	—	
Final outcome.....	D in 24 hrs.	R	R	D in 23 hrs.	

D = died.
R = recovered.

and those from younger cultures remain in the blood and within a short time bring about a fatal outcome, whereas those grown for a longer period in broth previous to injection are quickly removed, although they ultimately reappear and lead to death in every case. A brief

examination of Text-fig. 2 and Table II will show nevertheless that it is not until cultivation has proceeded for from 20 to 25 hours that much immediate clearing of the blood stream occurs. Even the cocci of a 16 hour and in some cases those of a 20 hour broth culture are for the greatest part still in a condition to resist the mechanism of the host,

ɪLE I

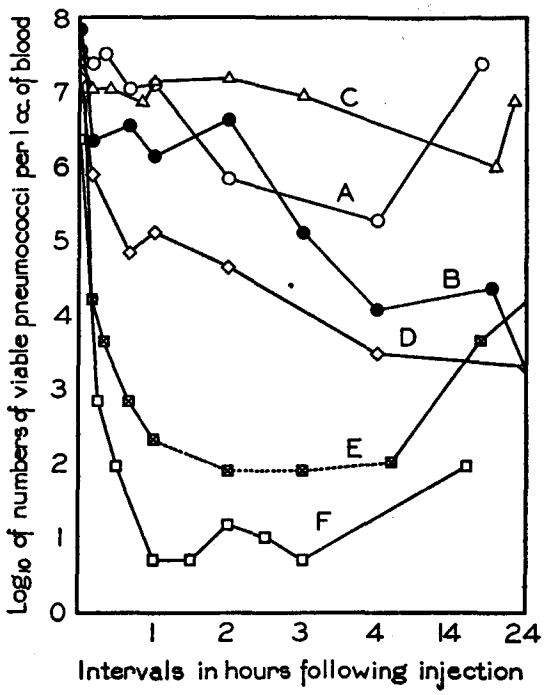
nal Rabbits with Strain CH Cocci
ɪle Pneumococcus Type III per Cubic Centimeter of Blood)

Source of inoculum					
sms from blood fected rabbit	Dextrose-serum broth cultures of varying ages				
it 18, infected od from No. 17, ig. 1, curve B)	Rabbit 2-76, 3 hr. culture (Text-fig. 1, curve C)	Rabbit 2-78 3½ hr. culture	Rabbit 14, 4½ hr. culture (Text-fig. 1, curve D)	Rabbit 9, 5 hr. culture (Text-fig. 1, curve E)	Rabbit 1, 10 hr. culture (Text-fig. 1, curve F)
10^7	2.1×10^7 (4 min.)	2.6×10^7	1.5×10^7	8.3×10^6 (3 min.)	2.3×10^6
10^6	—	1.1×10^7	7.8×10^5	1.6×10^4	680 (15 min.)
—	9.2×10^6 (15 min.)	1.1×10^7 (25 min.)	—	4.3×10^3	—
10^6 (40 min.)	3.1×10^6 (30 min.)	7.2×10^6 (40 min.)	7.0×10^4 (40 min.)	700 (40 min.)	90 (30 min.)
10^6	2.5×10^6	1.3×10^7	1.3×10^5	200	5
10^6	1.8×10^6	1.5×10^7	4.6×10^4	<100	5 (1½ hrs.)
					15 (2 hrs.)
10^5	4.1×10^5	9.0×10^6	—	<100	10 (2½ hrs.)
					5 (3 hrs.)
10^4	—	—	3.0×10^2	—	—
—	2.9×10^4	—	—	100 (6 hrs.)	—
10^4 (19 hrs.)	—	1 × 10^6 (20 hrs.)	—	4.5×10^3 (18 hrs.)	90 (16 hrs.)
10^3 (25 hrs.)	1.8×10^4 (22 hrs.)	7.9×10^5 (22 hrs.)	2.0×10^3 (24 hrs.)	—	—
—	—	—	—	2.3×10^4 (26 hrs.)	—
—	2.4×10^3 (46 hrs.)	—	150 (48 hrs.)	3.0×10^3 (42 hrs.)	22 (44 hrs.)
ırs.)	1.7×10^2 (69 hrs.)	—	—	300 (69 hrs.)	—
—	—	—	—	30 (92 hrs.)	—
—	—	—	—	2 (116 hrs.)	—
				0 (136 hrs.)	
R	R	D in 26 hrs.	R	R	R

which is remarkably efficient in removing the organisms in a 25 or 30 hour culture.

If these results be compared with the data recorded in Paper I concerning the relative times at which capsular diminution and loss

begin, it will be found that there is in general a close agreement between the onset of such changes in the organisms as cultivation is prolonged and the initiation of their extensive removal from the blood. Because



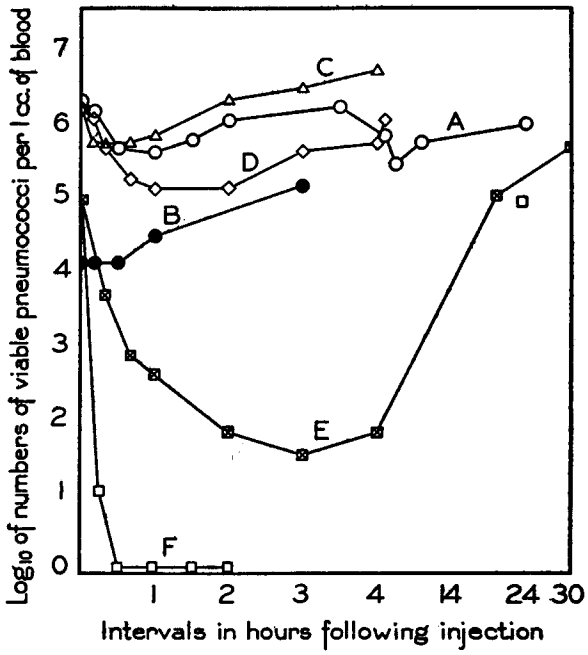
TEXT-FIG. 1. Curves illustrating the course of the initial bacteremia following the intravenous injection into rabbits of strain CH in various states of encapsulation.

Curve A, organisms from mouse peritoneum.
" B, organisms in the blood of an infected rabbit.
" C, organisms from 3 hour broth culture.
" D, organisms from 4½ hour broth culture.
" E, organisms from 5 hour broth culture.
" F, organisms from 10 hour broth culture.

of this correlation between capsular shrinkage and removal from the blood, we conclude that these phenomena are interdependent.

The experiments have a further importance in our study since they demonstrate the fact that if the rabbit avirulent strain is injected when the capsule is intact, such as occurs in the case of "animal" organisms

or those in a 3 hour culture, it may not infrequently lead to the death of the animal, providing the number of organisms in the blood can be maintained at a high level—an outcome never observed when older,



TEXT-FIG. 2. Curves illustrating the course of the initial bacteremia following the intravenous injection into rabbits of strain SV in various states of encapsulation.

Curve A, organisms from mouse peritoneum.
" B, organisms in the blood of an infected rabbit.
" C, organisms from 6 hour broth culture.
" D, organisms from 16 hour broth culture.
" E, organisms from 25 hour broth culture.
" F, organisms from 30 hour broth culture.

partially decapsulated cocci, even in very large doses, have been used. We have noted, however, that the organisms observed in smears of the blood taken during the course of the bacteremia or postmortem possess much smaller capsules in the case of CH than those of SV.

Particular significance must be assigned to the persistence in the

blood of 16 hour cultures of the rabbit virulent strain, since Wright (4) noting the failure of young cultures of Pneumococcus Type I to disappear from the blood stream, concluded that this depended upon their existing in the logarithmic phase of growth. It is apparent from the growth curves presented in Text-fig. 1 of the first paper that, after 16 hours, strain SV has been in the stationary period for about 6 to 8 hours. Its resistance to removal cannot therefore be associated with

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Intravenous Inoculation of Norm
Results of Blood Cultures (Expressed as Numbers of Viabl

Time interval elapsing between infection and blood culture	Organisms from mouse peritoneum	Infected blood transferred from rabbit 5-04			
	Rabbit 5-04 (Text-fig. 2, curve A)	Rabbit 5-05 (Text-fig. 2, curve B)	Rabbit 5-03 5 hr. culture	Rabbit 4-97 5½ hr. culture	Rabbit (Text-f
min.					
1	1.8×10^6	1.2×10^4	4.8×10^5	2.1×10^5	$1.4 \times$
10	1.3×10^6	1.2×10^4	4.6×10^5 (6 min.)	1.6×10^5	$5.1 \times$
20	—	—	3.2×10^5 (12 min.)	1.3×10^5 (25 min.)	$4.9 \times$
30–40	4.2×10^5 (30 min.)	1.2×10^4 (30 min.)	4.5×10^5 (30 min.)	9.0×10^4 (40 min.)	$5.0 \times$
hrs.					
1	3.7×10^5	2.8×10^4	8.3×10^5	1.6×10^5	$6.3 \times$
2	5.6×10^5 (1½ hrs.) 6.0×10^5 (2 hrs.)	—	1.2×10^6 (2 hrs.) 2.0×10^6 (2½ hrs.)	2.9×10^5 (2 hrs.) 5.4×10^5 (2½ hrs)	$1.8 \times$
3	1.5×10^6 (3½ hrs.)	1.3×10^5	—	7.3×10^5	$2.7 \times$
4	—	—	—	—	$4.7 \times$
5	6.1×10^5	—	—	—	
6	2.6×10^5 (6½ hrs.)	—	—	—	
10–20	4.3×10^5 (10 hrs.)	1.6×10^9 (16 hrs.)	—	—	
21–26	—	—	—	—	
27–40	—	—	—	—	
41–70	—	—	—	—	
71 hrs. onward	—	—	—	—	
Final outcome.....	D in 24 hrs.	D in 15¾ hrs.	D in 22 hrs.	D in 17 hrs.	D in <

the logarithmic phase of growth, but rather with the presence of capsule. We wish to point out that a lag period is observed following the introduction into the animal of organisms in any condition of growth, which we consider to be induced by alteration of environment. The duration of this lag *in vivo* is, however, shortest when the organisms in the phase of increase in the blood of one animal are transferred to another (Text-

fig. 2, curve B), since probably, under these circumstances, the change in environment is minimal.

Difference in Virulence of Strains SV and CH in Mice Following Intravenous Injection

The differences revealed between the rabbit virulent strain SV and the rabbit avirulent strain CH by the studies reported in Paper II,

E II

al Rabbits with Strain SV Cocci
e Pneumococcus Type III per Cubic Centimeter of Blood)

Source of inoculum

Dextrose-serum-broth cultures of varying ages

5, 6 hr. culture g. 2, curve C)	Rabbit 4-02, 16 hr. culture (Text-fig. 2, curve D)	Rabbit 18 20 hr. culture	Rabbit 31 21 hr. culture	Rabbit 32, 25 hr. culture (Text-fig. 2, curve E)	Rabbit 23, 30 hr. culture (Text-fig. 2, curve F)
10^6	1.3×10^6	1.9×10^6	1.0×10^6 (2 min.)	8.9×10^4	8.5×10^4
10^5	1.1×10^6	1.0×10^6	4.6×10^4	—	10 (15 min.)
10^5	4.2×10^5	8.7×10^5	5.2×10^3	4.3×10^3	—
10^5 (40 min.)	1.6×10^5 (40 min.)	6.6×10^5 (40 min.)	850 (40 min.)	670 (40 min.)	0 (30 min.)
10^5	1.2×10^5	4.0×10^5	240	360	0
10^6	1.2×10^5	1.8×10^5	0	60	0
10^6	3.8×10^5	3.6×10^5	0	30	—
10^6	4.9×10^5	4.8×10^5	0	60	—
—	1.0×10^6	1.6×10^6	—	—	—
—	1.1×10^6	2.2×10^6	—	—	—
—	—	—	1.4×10^4 (20 hrs.)	9.5×10^4 (20 hrs.)	—
—	—	—	2.7×10^4 (24½ hrs.)	—	7.5×10^4 (23½ hrs.)
—	—	—	9.3×10^4 (28 hrs.)	4.3×10^5 (30 hrs.)	—
—	—	—	1.7×10^5 (44½ hrs.)	1.3×10^9 (48 hrs.)	1.2×10^4 (48 hrs.)
			4.3×10^5 (51½ hrs.)		1.9×10^4 (69 hrs.)
—	—	—	—	—	4.4×10^3 (92 hrs.)
<18 hrs.	D in <24 hrs.	D in <18 hrs.	D in <66 hrs.	D in 48 hrs.	D in 117 hrs.

together with the experiments just recorded, made it seem improbable that their contrasting behavior in the rabbit was mainly attributable to any specific factor of the host. Since in the majority of Tillett's experiments and in our own, cultures were inoculated intravenously into rabbits, it seemed not unlikely that if the same route were adopted in the case of mice, variation in virulence might be revealed.

Accordingly, a number of series of white mice were injected into one of the caudal veins with 0.2 cc. of 6 hour dextrose serum broth cultures of each strain appropriately diluted in sterile broth. Counts of the viable organisms present in each culture were obtained by duplicate platings of 0.1 cc. of the higher dilutions injected. In Table III are summarized the combined results of several titrations made at different times. Although the numbers of mice employed are doubtless too few to give the results statistical validity, nevertheless, we consider that the virulence of strain SV for these animals is probably about 10,000 times that of strain CH. Data obtained using the intraperitoneal route indicate that there is a possible three- to fourfold difference in virulence when this method of inoculation is adopted.

These experiments suggest that, even in the highly susceptible mouse, the rabbit avirulent strain, when introduced in a manner permitting

TABLE III

Titrations of Virulence of Strains SV and CH in Mice Following Intravenous Inoculation

Strain	Nos. of pneumococci intravenously inoculated													
	10^{0} to 9×10^{0}		10^{1} to 9.9×10^{1}		10^{2} to 9.9×10^{2}		10^{3} to 9.9×10^{3}		10^{4} to 9.9×10^{4}		10^{5} to 9.9×10^{5}		10^{6} to 9.9×10^{6}		10^{7} to 9.9×10^{7}	
	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D
SV	1	0	7	9	2	14	1	15	nd		nd		nd		nd	
CH	nd		nd		nd		6	0	4	1	6	6	2	9	1	16

S = survived for 1 week or longer.
D = died.
nd = not done.
Figures under letters indicate numbers of mice.

the normal defensive mechanism of the body to function effectively, is definitely less virulent than the rabbit virulent strain. The former, however, may cause the death of both rabbits and mice, if the dose is sufficiently large, and in the case of rabbits, at least, if injected when completely capsulated.

Having established the facts that disappearance from the rabbit's blood stream of both strains went *pari passu* with capsular loss, and that difference in virulence appeared to be related to differences in the organisms themselves, and not to the particular species employed for testing this property, we proceeded to investigate, by means of

various techniques, the mechanisms by which removal from the blood stream is accomplished, again with the purpose of demonstrating differences which might be related to the changes in the surface properties of the organisms which have previously been described.

Phagocytosis by Fixed Tissue Cells

Since it is well known that after intravenous injection followed by the removal of organisms from the blood they accumulate in the sinusoids of the liver, in the splenic pulp, the capillaries of the lung and even in the muscles, we were led to an examination of stained sections of certain of these tissues from normal animals which had received large intravenous injections of the two strains, CH and SV. In general, the results obtained in a number of animals thus prepared corroborated those of the two experiments, the details of which are presented below.

Two rabbits, weighing 2.59 and 2.64 kilos, were injected with the centrifuged deposits from 45 cc. and 30 cc. of 15 hour blood broth cultures of strains SV and CH, respectively. Blood cultures were taken after ½ minute and 10 minutes. The organisms in the blood of the animal receiving strain CH were reduced from 1.38×10^8 per cc. to 3.72×10^4 per cc., while in the case of strain SV the counts were 3×10^8 per cc. after ½ minute, and 1×10^8 per cc. after 10 minutes. Immediately after the last blood culture, the animals were killed by fracture of the cervical vertebrae, and portions of the organs fixed in Zenker's solution. From these stained sections were prepared.

Study of the sections revealed in the liver of the rabbit inoculated with strain SV moderate numbers of organisms, the majority of which were extracellular, lying free in the sinusoids. They appeared to possess a well defined capsule. A few cocci were seen located within the Kupffer cells, apparently having no capsules. In the spleen of this animal, again, only moderate numbers of organisms were observed. It was difficult to be certain in all cases as to their relation to the cells, but in many instances they were lying free in the blood spaces. Capsulation was noted. After prolonged examination of the lung, only one extracellular short chain of cocci was seen. In the bone marrow likewise only one chain was observed, lying free in the lumen of a blood vessel.

In another experiment, 28 hour broth cultures of SV were injected into three rabbits which were killed after 10 minutes, 2½ hours and 4 hours. Unlike those in a 15 hour culture, most of the organisms injected were quickly removed from the blood stream of each animal. Examination of sections of the organs showed pneumococci in the Kupffer cells of the liver of the rabbit killed after 10 minutes and some in the phagocytic cells of the spleen. No extracellular cocci were

observed. In these organs from the animals killed after 2½ and 4 hours, practically no organisms—either intra- or extracellular—were seen.

The liver of the rabbit injected with strain CH revealed in certain areas large numbers of noncapsulated cocci, all of which appeared to lie within the Kupffer cells. As many as eight to nine cocci were counted within one Kupffer cell. Certain organisms stained poorly, contrasting with the intense coloration of others, suggesting in the former the onset of degenerative changes. In an occasional instance, it appeared that organisms had been taken up by polymorphonuclear leucocytes which themselves had been ingested by the Kupffer cells. Fewer cocci were observed in the spleen of this animal than in that of the rabbit injected with strain SV. Again, it was difficult to decide in every case concerning the intra- or extracellular position of the cocci, but some of them could be seen definitely within cells. Examination of the lung and bone marrow revealed in each only two diplococci within a polymorphonuclear leucocyte.

Upon examination of the organs of animals sacrificed after 7 hours or longer, in some cases only a very few and in others no organisms were seen.

From these and additional experiments of the same kind, it would appear that the majority of the organisms of strain SV, possessing as they do a capsule in a 15 hour culture, remained invulnerable to phagocytic action of the fixed tissue cells, particularly those of the liver. A minority which presumably had lost their capsules were taken up by such cells. The organisms from 28 hour cultures, many of which have lost their capsules, are rapidly ingested. The active phagocytosis of 15 hour cultures of strain CH, especially by the Kupffer cells of the liver, contrasts sharply with the behavior of strain SV at this age. Since it has been shown that in such CH cultures practically all of the capsule has been lost, we are again inclined to associate their susceptibility to phagocytosis by these cells as directly dependent upon the loss of this structure. In addition, the study of the sections suggests that the clearing of the blood is mainly effected by the fixed tissue phagocytes, although a certain number of organisms may be engulfed by polymorphonuclear leucocytes.

Fate of Organisms after Ingestion by Fixed Tissue Cells

It has thus been shown that the two strains disappear from the blood and are taken up by fixed tissue cells to an equal extent, provided the state of the organisms as regards lack of capsular material is the same, a condition obtained only with SV cultures of greater age. In the experiments under consideration here, we have sought to

determine whether ingestion by these cells leads to the destruction of both strains, or whether in one the bacteria may be killed and in the other survive and increase within these cells.

Counts were made of the viable bacteria in suspensions prepared from weighed portions of the organs from a series of rabbits killed at various intervals ranging from 10 minutes to 8 hours after intravenous infection. The counts were compared with those obtained from blood removed immediately before death. Rabbits were injected with the centrifuged suspensions from 30 to 40 cc. of blood broth cultures of the two strains taken up in a small quantity of sterile broth. 12½ to 15 hour cultures of strain CH and 15 hour and 28 hour cultures of strain SV were employed. In each case, the blood was cultured within ½ to 2 minutes following inoculation and again just before the animals were killed by a sharp blow over the occiput.

From the data obtained in representative experiments assembled in Table IV, it is evident that when there is only a slight diminution in the numbers of organisms in the circulating blood during the interval between injection and death, the ratio obtained by dividing the bacterial count for 1 gm. of liver, spleen or lung by that for 1 cc. of blood drawn immediately before the animal was killed is small, indicating that the organisms cultured from the organ sample are chiefly in the contained blood. This is exemplified by the rabbit injected with 15 hour SV organisms and killed within 10 minutes. When, however, a large decrease in the number of organisms occurs in the blood, as is apparent with the animals inoculated with a 28 hour culture of SV or a 15 hour culture of CH, and killed after 10 minutes, this ratio markedly increases, reflecting the accumulation of bacteria in the phagocytic cells of these organs. If the time of death be postponed for from 2 to 4 hours, the ratios obtained with 13 hour CH and 28 hour SV cultures again diminish and are roughly of the same magnitudes as those found for 15 hour SV. We believe therefore that in these animals killed from 2 to 4 hours after injection, which show low ratios, the organisms cultivated from the organs in greater part at least were present in the contained blood, and that those which had been taken up by the fixed tissue phagocytes were destroyed during this period.

Additional evidence for this view is found in the results of similar experiments, not presented in detail, in which ratios were recorded for 13 hour CH, when the animals were sacrificed after 40 minutes and 1 hour subsequent to injection, that

are intermediate between those for the rabbits killed after 10 minutes and 2 hours. Thus, the 40 minute and 1 hour ratios for liver to blood were 120 and 12 respectively. It will be seen that the ratio for 28 hour SV is still large at the end of 2½ hours, whereas before this time that of 13 hour CH has fallen to a minimum. The explanation probably lies in the fact that the 28 hour SV culture employed was removed from the blood somewhat more slowly than the 13 hour CH. Thus,

TABLE IV

Relative Numbers of Pneumococcus Type III in Tissues and in Blood at Varying Intervals after Intravenous Inoculation

Material cultured	Rabbit 1 killed after 10 min. Strain SV (15 hrs.)		Rabbit 2 killed after 10 min. Strain CH (15 hrs.)		Rabbit 3 killed after 1 hr. 50 min. Strain CH (13 hrs.)		Rabbit 4 killed after 8 hrs. Strain CH (13 hrs.)	
	Log. No. Pn*	Ratio†	Log. No. Pn	Ratio	Log. No. Pn	Ratio	Log. No. Pn	Ratio
Blood immediately after inoculation	7.917	—	8.140	—	9.328	—	9.226	—
Blood before death	7.586	—	4.571	—	5.306	—	5.602	—
Liver	6.594	0.1	7.328	570.0	4.462	0.15	4.538	0.098
Spleen	7.422	0.67	5.980	26.0	5.803	3.2	6.305	5.0
Lung	6.288	0.05	5.154	3.0	3.000	0.005	4.516	0.082
Material cultured	Rabbit 5 killed after 8 hrs. Strain CH (13 hrs.)		Rabbit 6 killed after 10 min. Strain SV (28 hrs.)		Rabbit 7 killed after 2 hrs. 10 min. Strain SV (28 hrs.)		Rabbit 8 killed after 4 hrs. Strain SV (28 hrs.)	
	Log. No. Pn	Ratio	Log. No. Pn	Ratio	Log. No. Pn	Ratio	Log. No. Pn	Ratio
Blood immediately after inoculation	8.493	—	7.256	—	6.977	—	5.414‡	—
Blood before death	4.105	—	4.681	—	2.114	—	2.684	—
Liver	3.398	0.25	5.987	20.2	3.881	60.0	3.042	2.38
Spleen	4.267	1.9	6.021	21.9	4.820	507.0	3.279	3.94
Lung	3.000	0.1	3.903	0.167	2.699	3.85	2.000	0.21

* Log. No. Pn = logarithm of the number of pneumococci per 1 gm. of tissue or 1 cc. of blood.

† Ratio = $\frac{\text{Number of pneumococci per 1 gm. of tissue}}{\text{Number of pneumococci per 1 cc. of blood}}$

‡ Blood taken after 3 minutes.

the ratio increases up to 2 hours after injection, denoting the storage of still viable cocci in the organs. By the end of 4 hours, however, these values have dropped to the low figures approximating those earlier found for CH which, as already noted, we consider to be indicative of the destruction of most of the cocci.

From these results we may conclude that when the organisms of both rabbit virulent and avirulent strains are taken up by the phagocytic

cells of certain organs, the majority are thereafter destroyed within about 2 hours. There is no evidence indicating any capacity on the part of the rabbit virulent strain to resist the bactericidal action of these cells once the integrity of the capsule has been impaired and an intracellular position has been assumed.

Phagocytosis by Polymorphonuclear Leucocytes

Tillett, in his studies on the immunity of rabbits to smooth Type III pneumococcus, failed to find evidence for the phagocytosis of this organism by polymorphonuclear leucocytes, whether or not it was virulent for these animals. He employed a method involving the use

TABLE V

Phagocytosis of CH and SV of Varying Age by the Normal Polymorphonuclear Cells and Serum of Rabbits and Mice

Source of cells and serum	Strain	Age of culture	Average No. of cocci per 10 cells	Cells containing organisms	No. cells counted
		hrs.		*per cent*	
Rabbit A	CH	6	5.1	9.0	200
	SV	6	0.04	0.02	500
	CH	16	87.0	86.0	50
	SV	16	13.7	30.0	100
Mouse A	CH	22	69.0	81.0	300
Mouse B	CH	22	11.2	33.0	262
Mouse C	CH	22	30.0	55.0	250

of a vital stain and direct observation of mixtures of serum, leucocytes and bacteria on a warm stage. His failures, together with our observations noted above of occasional cocci in this type of leucocytes in sections of tissues, prompted a further investigation of this phenomenon.

Suspensions of strains CH and SV from dextrose serum broth cultures of varying ages were added to mixtures of the exudative leucocytes and the defibrinated blood of normal rabbits, and rotated in sealed tubes at 38.5°C. Smears of the contents were made after ½ hour and counts of the organisms within polymorphonuclear leucocytes carried out. These, summarized in Table V, clearly demonstrate a considerable ability on the part of the rabbit leucocytes to ingest both strains, provided cultures of a sufficient age are employed. As was found when they were exposed to the action of human defibrinated blood (*cf.* Paper II),

there is a disparity between the time at which the organisms of the two strains become susceptible to the attack by the leucocytes. Thus, about seventeen times as many CH organisms were taken up by leucocytes when derived from a 16 hour broth culture as when the culture is 6 hours. Even in the latter, there is definite phagocytosis, however, whereas with a 6 hour culture of strain SV the numbers found in leucocytes are practically negligible. After 16 hours cultivation of this strain, however, a certain amount of phagocytosis is observed.

It was also possible to demonstrate *in vitro* considerable activity of the polymorphonuclear leucocytes of the mouse against older cultures. In one experiment, the details of which are also recorded in Table V, the exudative leucocytes from the peritonea of three mice, mixed with the homologous defibrinated blood, exhibited definite ability to take up a suspension of CH organisms from a 22 hour broth culture. It is interesting to note, in view of the well known variation in the susceptibility of normal mice to pneumococcus, that the numbers of organisms phagocyted by the cells and serum of the individual mouse are significantly different in each case. 10 hour cultures of this pneumococcus strain were definitely more resistant to phagocytic action although a small number were seen within cells.

These experiments then make it clear that the cells and serum of the normal rabbit and even the very susceptible mouse can bring about the ingestion of virulent Pneumococcus Type III. One of the factors which determines the extent to which this is accomplished is the degree of decapsulation of the cocci.

Phagocytosis by Exudative Leucocytes in Vivo

Since it was definitely established that polymorphonuclear leucocytes of the rabbit and mouse could, in the test tube, ingest large numbers of Pneumococcus Type III, but that this depended upon the state of the culture employed, we proceeded to determine whether or not these cells when present in an inflammatory exudate within the body behaved in the same manner. For this purpose an exudation of cells was produced in the peritoneal cavities of mice, and in the pleural cavities of rabbits by injections of sterile saline or broth as described in the section on technique.

1. In the Mouse.—Studies of smears prepared from the peritoneal exudate of normal mice removed 30 minutes after injection of suspensions of organisms of various ages obtained from broth cultures have shown that, in general, the results secured *in vitro* using mixtures of cells and serum were reproduced in the living susceptible animal. From the data presented in Table VI, it is apparent that if the culture inoculated be sufficiently old, the polymorphonuclear leucocytes of

the mouse are capable of taking up considerable numbers of both the rabbit virulent and avirulent organisms. Young cultures of the latter are much more resistant to phagocytosis, although it is evident that a small proportion of even 4½ hour culture of this strain may be ingested, while 4½ and 6 hour cultures of strain SV appear to be completely resistant. After a growth period of 12 hours, strain CH exhibits marked susceptibility to phagocytic attack, contrasting in this respect with the behavior of SV organisms of the same age, some of which, nevertheless, have now become definitely vulnerable. Although in certain instances, cocci were observed within large mononuclear cells, the majority were found in polymorphonuclear leucocytes.

These experiments serve to emphasize the importance of the relationship between the conditions at the surface of the organism at the

TABLE VI

Phagocytosis of Strains CH and SV by Polymorphonuclear Leucocytes in the Peritoneal Cavity of Mice

Strain	Age of culture	Average No. of cocci per 10 cells	Cells containing organisms	No. cells counted	Remarks
	hrs.		*per cent*		
CH	4½	0.8	2	50	
SV	4½	0.0	0	50	Further examination of preparation showed no phagocytosis
CH	6	2.25	8	40	Capsules on most extracellular cocci
SV	6	0.0	0	50	Capsules on most extracellular cocci
CH	12	92.0	80	25	Some cocci in large mononuclears—majority in polymorphs. No capsules on majority of extracellular cocci
SV	12	11.0	26	50	Phagocytosis observed only in polymorphs. Capsules on most extracellular cocci

time it is introduced into the body and the capacity of the local defensive mechanism of the host to attack it.

2. In the Rabbit.—Singer and Adler (5), in their studies on the immunity of the rabbit against Pneumococcus Type III, failed to observe any phagocytosis by polymorphonuclear leucocytes in pleural exudates. These authors, noting the presence of pneumococci within endothelial cells not only in the pleural cavity but in bone marrow and elsewhere, concluded that their elimination from the rabbit was exclusively dependent upon the phagocytic properties of the cells of the reticulo-endothelial system. Since there appeared to be a discrepancy between their findings and our demonstration of definite phagocytosis of both strains CH and SV by rabbit polymorphonuclears *in vitro* and by analogous cells in the peritoneum of the mouse, it became of interest to repeat the experiments of Singer

and Adler, using the two strains which have been studied. Furthermore, we wished to follow locally the course of events in the pleural exudate of rabbits until death or recovery occurred, with the object of correlating the state of the pneumococcus in respect to changes in the capsule with the degree of phagocytic activity should this be shown to occur.

In Table VII are summarized the observations made in the cases of two rabbits prepared by preliminary intrapleural injection of broth. One was inoculated with a 23 hour culture of strain CH and the other with a SV culture of the same age. An examination of the data will show that within 17 minutes after the injection of organisms in this late stage of growth, active phagocytosis took place in which both polymorphonuclear and mononuclear cells shared. This process was sufficiently effective to lead, within an hour, to a marked reduction in the numbers of extracellular cocci, which was reflected, at least in the case of strain CH, by about a ninefold reduction in the count of viable organisms present in the exudate. This fact, together with the striking decrease in the numbers of intracellular cocci of both strains observed after 1 hour, affords a certain amount of evidence for believing that the large proportion of the organisms originally introduced, which were seen within cells after 17 minutes, were rapidly killed and digested. We found no evidence of intracellular multiplication corresponding to that of Goodner and Miller (6), who believe that in the peritoneum of the normal mouse the pneumococci (Type I) ingested by the leucocytes multiply within the cell, ultimately causing it to rupture and in this manner are again liberated. By the end of 2 hours little change occurred in the exudate containing strain CH except that there was some indication of beginning encapsulation without any increase in the number of viable organisms. On the other hand, a definite multiplication of strain SV had begun and the cocci exhibited well developed capsules. After 7½ hours, large numbers of capsulated organisms were seen in both exudates. The capsules were very much larger in the case of SV. There is evidence that a small fraction of strain CH had again become susceptible to ingestion by leucocytes. This proportion of susceptible CH organisms was greatly enlarged by 21½ hours, although there were still many extracellularly situated. In the case of SV there was little evidence of phagocytosis at this time. Thereafter, until the death of the animal at 47½ hours, the extracellular encapsulated cocci were numerous and only a very few were seen within leucocytes. During the period of 49 hours following the onset of the marked phagocytosis of strain CH observed at 21½ hours, the numbers of viable organisms, which had been significantly reduced by 45 hours, remained more or less constant. Throughout this interval there was indication of great activity on the part of the leucocytes in taking up the organisms and, judging by the appearance of the intracellular cocci, in destroying them. Subsequently, the number of organisms within cells diminished, but complete sterilization of the exudate was not obtained until 94 hours later. In another similar experiment involving strain CH, no viable organisms were found in the exudate over a period extending from 44 hours to 90 hours after injection. At 120 hours, great numbers of encapsulated pneumococci were seen. 20 hours later the exudate became

TABLE VII

Phagocytosis of Pneumococcus III within the Pleural Cavity of the Rabbit

Time after infection at which exudate withdrawn	Strain CH		Strain SV	
	Remarks on microscopic examination of stained exudate	No. organisms per 1 cc. of exudate	Remarks on microscopic examination of stained exudate	No. organisms per 1 cc. of exudate
min.				
17	Marked phagocytosis by polymorphs and mononuclears. Moderate numbers of extracellular cocci	3×10^6	Considerable phagocytosis—more by mononuclears than by polymorphs but definite in latter. Small numbers extracellular noncapsulated cocci	Confluent growth from 0.005 cc.
hrs.				
1	Only 2 polymorphs and 2 mononuclears seen with ingested cocci. Very few pneumococci seen extracellularly	4×10^5	Only a few organisms seen outside cells; none within. Evidence of beginning encapsulation	Confluent growth from 0.005 cc.
2	Practically no evidence of phagocytosis. Extracellular cocci infrequent. Those seen had small capsules	2×10^5	No phagocytosis noted. Marked increase in number of extracellular organisms which have large capsules	1×10^8
7½	A few cocci in polymorphs and mononuclears. Many extracellular encapsulated cocci in short chains	Confluent growth on plating 0.01 cc. of exudate	No phagocytosis. Large numbers of extracellular cocci in chains having huge capsules	Confluent growth from 0.02 cc.
21½	Marked phagocytosis by polymorphs. Extracellular organisms numerous	9×10^6	A few cocci in mononuclears; none in polymorphs. Moderate numbers extracellular capsulated cocci seen	1.3×10^5
27	Failed to obtain exudate		No phagocytosis noted. Numerous extracellular encapsulated organisms	Confluent growth from 0.001 cc.
45	Very marked phagocytosis by polymorphs and monocytes. Intracellular organisms stain best by Gram and poorly by Wright. Indication of beginning digestion? Very few extracellular cocci	3.5×10^3	Some phagocytosis by mononuclears and to a lesser degree by polymorphs. Numerous extracellular encapsulated organisms	1.5×10^6
47½	Extensive phagocytosis; about 30 to 40 per cent of polymorphs contain cocci. As many as 10 to 20 may be seen in one cell. Gram stain better than Wright. Very few extracellular cocci	1×10^4	No definite phagocytosis. Large numbers of heavily capsulated, extracellular organisms. Animal died at this time	Confluent growth from 0.001 cc.
70½	Less phagocytosis than at 47½ hours. Cocci within cells appear to be disintegrated. No organisms seen extracellularly	1×10^5		
93¼	No phagocytosis by polymorphs. A few cocci in mononuclears. A few extracellular capsulated cocci; one chain in about 10 fields	Cultures contaminated		
140½	No phagocytosis. No extracellular organisms seen	9×10^3		
164	No phagocytosis. No extracellular organisms seen	0		

sterile, but smears stained by Gram revealed many intracellular, partially digested cocci.

We wish to point out that the changes in the bacterial population in the exudates parallel fairly closely the course of the bacteremia in animals intravenously inoculated with the two strains, as will be seen from the data recorded in Tables I and II.

In summary, it may be stated that the ultimate fate of these two strains in the pleural exudates of rabbits appears to depend upon the difference in their ability to maintain a resistance to ingestion by leucocytes after the initial decline and subsequent phase of increase has occurred. We consider that this difference in the organisms which have developed within the body is associated with the same properties that have been shown to account for their resistance or susceptibility to phagocytosis, when they have been cultivated in artificial media. It is not improbable that with strain CH growing in the exudate a balance is soon struck between the rate of production of young invulnerable forms and the rate at which changes take place at the surfaces of the cocci, which render them susceptible. In this manner the number of extracellular viable organisms is restricted and in some cases may be reduced almost to zero. Since in strain SV these changes proceed more slowly, no such balance is established within the short time that these animals survive. The numbers of resistant forms quickly come to enormously outnumber those which have lost most of their capsule and in consequence have been removed by leucocytes.

DISCUSSION

Up to the present no adequate explanation has been offered for the observed differences in the virulence for rabbits of various strains of Pneumococcus Type III. In Paper I of this series it was shown that smooth strains of the organism could be grouped on the basis of their ability to remain viable and to multiply at 41°C. or their lack of this quality. The majority of strains tested died off more or less rapidly at this temperature which is usually attained within a few hours after intravenous infection by normal rabbits. Such strains, when tested for rabbit virulence by this route, failed to kill. All of the virulent strains grew at 41°C. but some of the "thermo-resistant" strains were avirulent in the quantities injected. It appeared that the ability to

proliferate at 41°C. was a prerequisite but not the sole factor in determining the degree of virulence of a given strain. In an attempt to further analyze the problem we selected for study two strains which differ in their lethal properties for rabbits, but both of which are able to grow at 41°C. In Paper II we have determined that *in vitro* at 37°C. the avirulent strain CH produces a somewhat smaller capsule than the rabbit virulent strain SV and, furthermore, that the loss of the structure during cultivation which leads to surface changes giving the cells, at least temporarily, properties similar to those of the R organisms, occurs more rapidly in the former. In this paper it has been demonstrated that the extent of capsular disintegration at the time of injection is directly correlated with the capacity of the animal body to remove both strains from the blood and to eliminate them through ingestion and destruction by means of the phagocytic cells, chiefly those of the fixed tissues but to a certain degree by the mobile leucocytes as well. Furthermore, from an examination of the events transpiring within the pleural cavity subsequent to the introduction of the two strains, it appears that just as *in vitro*, so in the body, diminution of the smaller CH capsule (which at first speedily develops) takes place readily and advances swiftly, leading to extensive phagocytosis by polymorphonuclears. In contrast, the vast majority of SV cocci, after the production of their massive capsules, remain invulnerable in the presence of leucocytes during the period preceding the death of the animal.

In view of all the foregoing experimental data we feel justified in asserting that (*a*) the inherent physiological potentiality of the bacterium to grow in the body of the host under the environmental conditions, including elevated temperature, encountered following infection, (*b*) together with the ability of the organism to maintain the capsule intact for a longer or shorter period of time, are fundamental factors in determining whether the injection of a given strain of smooth Type III pneumococcus will ultimately result in an invasion of the host characterized by practically unlimited proliferation of the organisms and ending in death, or whether the disease will be marked by a restrained increase in the cocci reflected by a controlled, low grade, fluctuating bacteremia which is ultimately overcome.

There is no difficulty in perceiving the application of this statement

to the case of the virulent strain SV. Given the ability to proliferate rapidly in the environment of the host, the production and retention of a large capsule insure it against destruction by phagocytes and permit multiplication to proceed practically unhindered. In considering the behavior of CH we will recall that this organism while able to grow at 41°C. also dies off much more quickly at such temperature. This fact together with the rapid loss of the capsule may serve to explain why the cocci found in the blood during the phase of secondary bacteremia, and which in order to remain there must be equipped with capsules, do not multiply unchecked and overwhelm the animal. Complete sterilization of the blood stream, which usually occurs on the 5th or 6th day after infection, is probably brought about by the development of antibodies which may be type specific (unpublished experiments) or species specific (*cf.* Paper IV[1]).

The fundamental significance of temperature is further emphasized by the information presented here. The phagocytes of the rabbit are able to attack the virulent or avirulent Type III pneumococci, but only on the loss of their protective envelope. Since nearly all of the strains in our group 3 (*cf.* Paper I) which were susceptible to the deleterious effect of elevated temperatures possessed very large capsules, sometimes exceeding in size those of SV and retained *in vitro* for long periods, one might have anticipated that they would have remained nonphagocytizable by the cells of the host and therefore highly lethal. The lack of virulence of these strains with very large capsules can only be attributed to their failure to grow in the host as a result of the hyperthermy developed and their removal from the blood stream following death. Strain Tirrell in group 2 (Paper I), which was also able to persist at 41°C., resembled CH in possessing capsules which (although slightly larger) were likewise speedily impaired, and its avirulence would appear to depend on this attribute. In contrast, strain IE with similar growth properties at 41°C. and possessing a very large persistent capsule proved highly virulent.

Although our study has been principally directed toward the analysis of the factors which account for the difference in virulence of Pneumococcus Type III strains in rabbits, we believe that these findings have a broader applicability since it has been shown that a similar distinction

[1] Enders, J. F., Wu, C.-J., and Shaffer, M. F., *J. Exp. Med.*, 1936, **64**, in press.

in virulence for mice is exhibited provided the intravenous route be employed. This fact affords further evidence that the attributes which determine virulence are to be sought in the bacteria themselves, rather than in the particular species of host. We cannot, therefore, agree with the hypothesis of Tillett, that the rabbit possesses a host specific mechanism which enables it to destroy the capsule of the avirulent Type III Pneumococcus, but not that of the virulent variety.

The importance of capsular retention by the Pneumococcus as it affects the removal of the organisms from the blood has not been generally recognized by previous workers in this field. Thus, Teale (7) finds that the young actively growing cultures of virulent Pneumococcus Type III in contradistinction to nearly all other species of bacteria are not rapidly eliminated by the normal clearing mechanism of the blood. He definitely asserts that this "is not due to a capsule interfering with phagocytosis since it is not present in cultures," but presents no alternative explanation. Wright, observing the similar behavior of young cultures of Type I Pneumococcus following intravenous inoculation into rabbits attributed their persistence in the circulation to the fact that they were in the logarithmic growth phase, which continued unchecked after introduction into the animal. We do not feel, at least in the case of Pneumococcus Type III, that this is due to the phase of active growth *per se*, but rather to the fact that the size of the capsule is greatest at this stage. This view is borne out by certain experiments with SV in which the organisms, although long past the logarithmic growth period, still possessed large capsules and were not removed following injection. Further, the injection of CH cultures still in the phase of active multiplication was followed by a marked diminution in the numbers of cocci in the circulating blood. Finally, we have found that even when SV organisms, which had previously been increasing rapidly in the blood of one animal, are transferred to another, there is a short but definite initial period during which no increment in numbers is observed.

SUMMARY AND CONCLUSIONS

Among the experimental findings reported in this paper to which we wish to give particular emphasis are the following:

1. The results which follow the intravenous injection into rabbits

of two strains of Pneumococcus Type III of different degrees of virulence vary with the state of the capsule. Thus when this structure is completely developed both remain in the blood. A culture of either strain begins to become susceptible to the blood-clearing mechanism contemporaneously with the onset of capsular degeneration and the initiation of other concomitant changes at the surface of the organism (*cf.* Paper II), which occur much earlier with the less virulent strain.

2. When, in either case, removal from the blood stream occurs, this is effected by the phagocytic cells of the body. There is no suggestion that a new or unknown mechanism is involved. The greatest share of the burden is borne by the fixed phagocytic cells of the liver and spleen, and to a less extent by those of the lung and bone marrow. Nevertheless, it has been demonstrated that the polymorphonuclear leucocytes may also participate.

3. Phagocytosis by the leucocytes of the normal animal either *in vitro* or *in vivo* has been observed only at such a time as the capsule has become impaired. Ingestion of the organisms by the fixed tissue cells appears also to be effective only under the same condition and is accordingly observed with much younger cultures of the less virulent strain.

4. Following their removal from the blood and their accumulation within the fixed phagocytes of the organs, destruction of most of the cocci proceeds within 2 to 4 hours. Both strains are destroyed provided they are in the state favorable to phagocytic attack.

5. Evidence has been presented which indicates that just as *in vitro*, so in a local area of inflammation within the body, aging with attendant capsular loss and increasing susceptibility to phagocytosis may take place.

6. With organisms from either strain a variable period of lag follows their injection into the blood stream, even when they are introduced in a state of active multiplication and complete encapsulation.

7. Differences in virulence for rabbits of two strains of Pneumococcus Type III do not imply that this animal possesses a defensive mechanism which is absent in other species, since it has been possible to demonstrate similar differences when the organisms are injected intravenously into mice. This fact indicates that the factors determining the degree of virulence of these strains are to be sought in the organisms themselves, rather than in the kind of host.

8. Differing degrees of virulence among various strains of Pneumococcus Type III capable of growing at the elevated temperature encountered in the infected animal appear to be conditioned by variations in the capacity to maintain the integrity of the capsule within the body.

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EXPLANATION OF PLATE 21

FIG. 1. Liver of rabbit injected with 15 hour broth culture of strain SV. Large encapsulated organisms lying free in sinusoid about a fragment of adventitious debris.

FIG. 2. Liver of same rabbit. Pneumococci in Kupffer cell; compare with Fig. 1, noting absence of capsule.

FIG. 3. Liver of same rabbit. Short chain of pneumococci apparently within a polymorphonuclear leucocyte lying in the sinusoidal space.

FIG. 4. Liver of rabbit injected with 15 hour broth culture of strain CH. Pneumococci in Kupffer cell. Note absence of capsule.