THE EFFECT OF INJECTIONS OF HEMOLYTIC STREPTOCOCCI ON SUSCEPTIBLE AND INSUSCEPTIBLE ANIMALS.

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The observations recorded in this paper were made in the course of a study of streptococcus infection and immunity. In order to study the problem of the immunization of animals against streptococcus infection, it was necessary first to obtain as clear a picture as possible of the course of the infection in untreated animals. It has been noted by other workers, and is borne out by our own observations, that bacteria introduced into the circulation of the living animals quickly disappear. It was our first task to study this phenomenon and if possible to find some means of explaining it.

We found that with hemolytic streptococci, which are of rather low virulence for rabbits, a sublethal dose may completely disappear from the blood stream within a few hours. If a lethal injection is given, over 90 per cent of the cocci are removed from the circulation within the first few minutes, and subsequent blood cultures reach a minimum in 2 to 3 hours, but after 4 to 6 hours the number again begins to increase. Even with lethal doses, cultures of 1 cc. of blood may show no colonies after 2 or 3 hours, but, as a rule, streptococci do not completely disappear after such an injection. The usual course of a primary removal is shown by the cultures taken after the first injection recorded in Experiment 2. An example of complete removal is shown in Experiment 1.

Experiment 1.—Rabbit injected with 5 cc. of a heavy suspension of Streptococcus 43. Cultures taken immediately showed several thousand organisms per cc. of blood. After 10 minutes, five drops of blood showed only 23, and after 30 minutes, 5; after 1 hour, 2 hours, 4½ hours, 11½ hours, and 26 hours, 1, 0, 6, 17, and 118 organisms respectively.

The blood cultures subsequent to those recorded in the experiment were consistently positive until the death of the animal on the 4th day. The prompt and almost invariable removal of organisms artificially introduced into the circulation has been difficult to harmonize with the fact that in susceptible animals the organisms again appear in the blood within 4 to 12 hours, and may be found constantly in the circulation until death, as in cases of spontaneous septicemia.

There are four hypotheses which might account for this: first, that after a period the blood-sterilizing mechanism becomes exhausted and permits bacteria to remain in the circulation; second, that the bacteria after a brief stay within the body become resistant to its defensive powers; third, that the injection of foreign protein stimulates some reaction such as a mobilization of antibodies, or of phagocytes, which would account for the prompt and extensive preliminary removal of the bacteria; fourth, that the bacteria do not multiply in the circulation, but are continually introduced from infected tissues faster than they can be removed.

The first possibility is practically ruled out by the fact that if, during the period of the reappearance of bacteria from the circulation, an additional number many times in excess of those present is introduced, they are removed as rapidly, or even more rapidly than after the first injection, and that this reinjection, and subsequent removal of the bacteria, may be repeated until the animal becomes moribund. This fact, originally observed by Bull,[1] is well illustrated in the following experiment:

Experiment 2. Removal of Streptococci after Repeated Injections.—A rabbit weighing 1,620 gm. was injected into the ear vein with a suspension of Streptococcus 5, containing 72,000 chains of small clumps per cmm. Five drops of blood from the opposite ear were plated in sheep blood agar at the intervals noted.

First Injection, 4.8 Cc.—Cultures taken after 1 minute showed 60,000 colonies; sank to 135, 3, and 5 after 10 minutes, 1 hour, and 4 hours respectively; rose to 1,800 at 6 hours; showed 127, 150, and 8 colonies after 23 hours, 25 hours, and 25 hours, 4 minutes respectively.

Second Injection, 25¼ Hours after First Injection. 2.4 Cc. Injected.—Cultures ½ minute after injection showed 62,000 colonies; sank to 19 after 10 minutes and remained low for 3 hours; after 4 hours cultures showed 200 colonies; after 7½ hours, 100; after 8 hours, 56.

[1] Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.

Third Injection, 8 Hours, 10 Minutes after Second Injection. 0.5 Cc. Injected.—Cultures ½ minute after injection showed 21,000 colonies; fell to 15 after 10 minutes; after 20 minutes, ½ hour, 1½ hours, 2½ hours, and 13½ hours, showed 180, 280, 75, 30, and 220 colonies, respectively; rose to 1,320 at 16½ hours; and after 20 hours, 21 hours, 23 hours, 11 minutes, and 23 hours, 27 minutes, showed 33, 51, 120, and 221 colonies, respectively.

Fourth Injection, 24 Hours, 28 Minutes after Third Injection. 4.9 Cc. Injected.—Cultures ½ minute after injection showed 65,000 colonies; sank to 90 after 11 minutes, and remained fairly low for 3½ hours; rose to 480, 4½ hours after injection. Died 20 hours after injection.

The second possibility, namely, that the streptococci have become resistant, deserves more careful observation. Considerable evidence has been brought forward to show that bacteria which have grown in the body differ strikingly in their resistance from the same organisms cultivated artificially. The studies of Gruber and Futaki[2] on anthrax, and of Bail,[3] Eisenberg,[4] and others on Gram-negative bacilli, are too familiar to require more than passing reference. In his studies on pneumococcus infection in dogs, Bull[1] has shown that the organisms which reappear in the circulation after the primary fall in sepsis are more resistant to the action of immune sera than those injected.

However, in the case of hemolytic streptococcus infection in the rabbit, this does not seem to offer a satisfactory explanation. It is true that streptococci which have multiplied in the peritoneal cavity of a mouse show a striking resistance to agglutination and phagocytosis, even in the presence of immune serum derived from rabbits, as compared with streptococci from culture. The latter, as a rule, agglutinate spontaneously, and are extensively phagocyted in salt solution, whereas suspensions from the peritoneums of infected mice have often failed to agglutinate in the immune sera in our possession, and show high relative resistance to phagocytosis. Nevertheless, if these bacilli from the mouse peritoneum are injected into a rabbit's circulation, they are disposed of almost as effectually as are culture organisms. This is illustrated in the following experiment:

[2] Gruber, M., and Futaki, K., *Münch. med. Woch.*, 1906, liii, 249.

[3] Bail, O., *Arch. Hyg.*, 1905, lii, 272.

[4] Eisenberg, P., *Centr. Bakteriol., Ite Abt., Orig.*, 1908, xlv, 638.

Experiment 3. Removal of Streptococci Obtained from Peritoneum of Mouse.—Three mice were injected intraperitoneally with Streptococcus 5. The 24 hour exudate was removed in citrate, centrifuged to remove leukocytes, then more rapidly to throw down the cocci. The second sediment was suspended in salt solution. A broth culture of the same organism was centrifuged and the sediment resuspended in salt solution. Two rabbits were injected into the ear vein and cultures of fifteen drops taken from the opposite ear. It is difficult to count accurately the number of organisms in the suspensions, and it is possible that the doses were unequal. Nevertheless, the prompt removal of nearly all the streptococci from the circulation is evident, though it would appear that the culture organisms were removed more rapidly than those from the mouse.

Rabbit 1, weight 1,010 gm., was given 10 cc. of streptococcus suspension from a mouse, containing 64,000 chains per cmm. Cultures at 5 minutes showed 2,000,000 colonies; at 10 minutes, 1,300; at 30 minutes, 900; at 1 hour, 103; at 3¾ hours, 257.

Rabbit 2, weight 980 gm., was given 10 cc. of streptococcus suspension from a culture containing 56,000 chains per cmm. Cultures at 5 minutes showed 1,000,000 colonies; at 10 minutes, 400; at 30 minutes, 60; at 1 hour, 24; at 3¾ hours, 192.

This experiment is open to the objection that the resistance acquired by streptococci growing in the peritoneum of a mouse might not be the same in degree or in kind, as that acquired in the circulation of the rabbit. It is technically impossible to introduce into a rabbit's circulation bacteria from the circulation of another rabbit comparable in number to those which can be introduced from cultures. However, the following experiment seems to show that a change in the streptococci during their multiplication in the animal is not the factor which determines their persistence in the circulation. A rabbit was given a large intravenous injection of organisms from cultures, and 48 hours later, when the organisms were numerous in the blood, and the animal was almost moribund, 20 cc. of blood were taken from the heart, under ether, into a cooled paraffined syringe and immediately injected into the ear vein of a normal rabbit. The blood used for injection contained 6,600 organisms per cc. Cultures taken from the second rabbit gave the following result:

Experiment 4.—Cultures taken from the second rabbit showed 2,400 per cc. 1 minute after injection; 600 after 3 minutes; sinking gradually to 108 at 2 hours; at 24 hours, 16 colonies; and at 48 hours, 0. The animal survived.

This seems to show that the streptococci which one finds in a so called septicemic rabbit have not acquired resistance sufficient to maintain them in the circulation.

In regard to the third possibility, a number of our earlier observations seemed to indicate that the injection of the bacteria, acting as a foreign protein, excited some reaction in the animal which caused their removal. On several occasions when a septicemic animal was reinjected with the same organisms, the bacterial count 1 or 2 hours after injection fell to a point distinctly below the count before injection. This seemed to indicate that, following the second dose, the animal got rid of not only all the newly introduced organisms, but also some of those previously in the circulation. Bull[1] has reported this same phenomenon in dogs injected with pneumococcus.

Experiment 5. Reduction in Sepsis Following Reinjection. First Injection. 3 Cc. Injected.—3 cc. of streptococci containing 55 million per cc. injected. After 1 minute culture of fifteen drops from opposite ear showed 22,000 colonies; fell to 95, 12, 2, 9, and 3, after 10 minutes, 30 minutes, 1 hour, 3½ hours, and 4½ hours after injection respectively.

Second Injection, 4 Hours, 20 Minutes after First Injection. 3 Cc. Injected.—Cultures showed 23,000, 1 minute after injection; 8, 2, and 936, 10 minutes, 43 minutes, and 18 hours after injection respectively.

Third Injection, 18 Hours, 20 Minutes after Second Injection. 5 Cc. Injected.—Cultures showed 3,000 colonies 1 minute after injection; 38, 32, 52, and 750, 10 minutes, 45 minutes, 2¾ hours, and 6 hours after injection respectively.

It will be noted that following the third dose, the greater number of the injected organisms was removed within 1 minute, as the first culture taken contained only 3,000 colonies as compared with 22,000 and 23,000 following the first and second injections. In 10 minutes after the third injection the count dropped to 38 as compared with 936 before the injection. In other words, not only did the organisms injected disappear from the blood stream, but the majority of those previously present in the circulation also disappeared, and it was not until more than 2¾ hours after the third injection that the sepsis again approached its previous intensity. A similar result was obtained following the second injection of Rabbit 4, Table I, and following the third injection recorded in Experiment 2.

The result could not be obtained regularly in experiments conducted in a similar way, but occurred with sufficient frequency to make necessary further investigation. As an explanation of this reaction there occurred to us the possibility that the injection of the additional number of streptococci caused a mobilization of antibodies, or, more probably, a sudden leukocytic reaction such as has been noted following the injection of specific and also of non-specific proteins into infected animals.

It seemed possible that in cases where the count after injection did not fall below that before injection the removal of the organisms already present in the blood might be masked by the large additional number injected. If the removal of the organisms from the blood following a second injection was due to a reaction by the body to the injected streptococci, acting as a foreign protein, it was probable that it could be reproduced by the injection of killed streptococci, of other species of bacteria, of serum, or of peptone. Should it occur after injections of such non-specific substances, it would appear more clearly, as the picture would not be confused by the growth of the newly injected organisms in cultures taken after injection. However, in a series of experiments in which the substances mentioned above were injected into septicemic rabbits, no consistent effect on the number of organisms in the circulation could be observed.

On further study it appeared that we had at first attached too great significance to these apparent remissions in the sepsis following reinjection. It will be noted in Experiment 2, that after the third injection the number of organisms in the circulating blood varied enormously within a few hours. These variations have been noted so often that we believe the possibility of their being due to technical error can be ruled out. The only influences brought to bear upon the animal during this period of variation were the withdrawal of small samples of blood from the ear and placing the animal head down in a warmed box to facilitate bleeding. We have carefully tested the effect on septicemic animals of repeated small bleedings, of maintenance for considerable periods in a vertical position, and of maintenance in a warm chamber, but none of these procedures had any constant effect on the course of the sepsis. As our observations accumulated, these variations continued to appear and to support

the idea, which we shall refer to again, that streptococcus rabbit sepsis is not a condition in which a definite number of organisms is constantly in the blood, but one in which organisms are continually swept into the circulation from heavily infected tissues.

The possibility that a leukocytosis was responsible for the removal of the organisms from the blood was definitely excluded by making blood counts. The effect of streptococcus injections on the leukocytes was not constant, but the usual reaction was an immediate leukopenia, sometimes rather marked, as illustrated by Rabbit 3, Table I. Repeated injections as a rule caused a still further fall of leukocytes, as illustrated by Rabbit 4, Table I. In a few instances after smaller injections, a transitory rise in the blood count, as illustrated in Rabbit 5, Table I, was observed, but this was exceptional and could not account for the removal of the injected bacteria which was invariable. Whether or not the leukopenia is usually followed by a leukocytosis we cannot state. In several animals studied we failed to observe it. We did not, however, make frequent counts for more than 6 hours after injection, as by this time the streptococci have not only been removed from the circulation, but, as will be seen later, have been largely killed, and a leukocytosis occurring later could have no bearing on the phenomena we were endeavoring to

TABLE I.

Rabbit 3. Leukopenia Following First Injection. 3 Cc. of Streptococcus Suspension.

Time.	Temperature.	White blood count.	Colonies per cc.
	°F.		
Before injection	102.8	9,000	
1 min. after	102.9	6,100	1,000
10 " "	103.0		27
½ hr. "	103.4	5,800	7
2½ hrs. "	105.4	3,100	
4 " "	105.8	2,600	4
1 day "	105.0	4,100	500
2 days "	105.4	6,100	43
3 " "	103.6	6,000	0
4 " "	105.4	9,400	0
5 " "	104.5	9,100	0
7 " "	105.0	15,000	1,170

TABLE I—*Concluded.*

Rabbit 4. Progressive Leukopenia Following Second Injection.

Time.	Temperature.	White blood count.	Colonies. per cc.
	°F.		
Before injection		10,800	
1 min. after 1st injection	102.6		1,000,000
10 " " 1st "		6,500	1,820
30 " " 1st "	105.1	8,200	37
1 hr. " 1st "	105.7	6,200	35
2½ hrs. " 1st "	104.2	7,200	
4½ " " 1st "	105.6	5,200	169
4 " 35 min. Reinjection.			
1 min. after 2nd injection			900,000
10 " " 2nd "			569
30 " " 2nd "	105.6	3,100	48
1 hr. " 2nd "	104.7	2,800	194
2 hrs. " 2nd "	103.6	500	214

Found dead 21 hours after 1st injection.

Rabbit 5. Transitory Leukocytosis Following Injection.

Time.	Temperature.	White blood count.	Colonies. per cc.
	°F.		
Before injection		6,800	
1 min. after			13,000
30 " "	101.9	11,400	336
2½ hrs. "	103.8	7,600	34
5 " "	103.6	4,700	315
12 " "		3,500	600

Found dead 22 hours after injection.

explain. The absence of a general leukocytosis does not, of course, exclude the possibility that in the visceral capillaries the leukocytes take part in the destruction of the invaders. This question will be taken up later.

To repeat, the injection of streptococci is not immediately followed by a leukocytosis and we have been unable by the injection of various non-specific substances to excite any reaction which would remove the bacteria from the circulation of a septic animal. These negative results do not exclude the possibility that the living streptococcus

itself can cause some such reaction. However, in view of the fact that the injection of additional streptococci does not regularly cause a reduction in the sepsis, and also of the fact that the reductions we have observed have all been within the range of spontaneous variations in such infections, we believe that a reaction of this sort can be reasonably excluded as an explanation for the primary removal of bacteria from the blood stream.

The fourth possibility, that the bacteria found in the blood of septicemic rabbits are not actively persisting in the circulation, but are merely swept into the circulation from infected tissues more rapidly than they can be removed seems to us the most probable explanation. This hypothesis has been commonly adopted for infections such as subacute bacterial endocarditis in man, where the septicemia is intermittent. While at first sight this supposition seems unlikely in the case of a continuous sepsis, the fact that in these animals the number of bacteria in the muscles and other organs far exceeds those in the blood, weight for weight, would at least explain the source of the constant supply of bacteria. We shall discuss this point more fully later.[5]

Mechanism of Removal.

The observations mentioned above show that even a susceptible animal, such as the rabbit, is able to remove from its circulation almost any number of introduced streptococci, even after successive injections and until a lethal condition has developed, and will almost or completely free its circulation from the organisms within a few hours unless a new supply is reintroduced. Concerning the mechanism of this removal and the ultimate fate of the bacteria, however, there has been considerable discussion.

Three factors have been suggested as entering into this removal of bacteria: agglutination, phagocytosis by leukocytes, and phagocytosis by fixed cells. We have looked for evidence, such as that reported by Bull, of agglutination in rabbits and in cats. The organisms as injected, even though previously shaken for a long time with glass beads, often show clumps of from two to ten chains. Clumps of this

[5] We are indebted to Miss B. H. Paige for assistance in the blood culture studies.

size have naturally been found in the blood and in the organs, but seldom have they exceeded the diameter of the average capillary. We have also seen groups of streptococci of about the size and contour of a leukocyte sometimes entangled with leukocytic remnants. These we have interpreted as being groups of organisms which had been taken up by leukocytes which have subsequently disintegrated. Aside from these small groups of cocci which do not resemble in the least the felt-like masses of streptococci which have agglutinated *in vitro* we have seen neither in films of the blood nor in crushed preparations or sections of the organs evidence of agglutination, and it seems unlikely that it plays a part in the phenomenon we have studied.

In regard to the leukocytes, these cells undoubtedly take up injected streptococci in considerable numbers, and with great rapidity, as can be seen a few minutes after injection in blood films or in sections, and it has been frequently claimed that these cells play a predominating part in the removal of microorganisms from the blood. Wyssokowitsch,[6] however, found the bacteria lying apparently in endothelial cells, and recently Kyes[7] has demonstrated in the pigeon the taking up of pneumococci by the Kupffer cells of the liver, and by endothelium in other organs of the body. Manwaring and Coe[8] have analyzed this phenomenon further by carefully washing livers free from blood and then perfusing the organs with virulent pneumococci and other organisms. They found that whereas virulent pneumococci treated with immune serum were quantitatively removed by the blood-free liver, these organisms when suspended in Ringer's solution or in normal serum were not removed even by livers of immunized animals, or were taken up to a slight extent. This they attributed to the action of traces of serum which had not been removed from the organs, and they speak of the immune substance to which this is due as "endothelial opsonins."

Bartlett and Ozaki,[9] in studies on dogs injected with staphylococci, noted the primary accumulation in the lungs, but describe the leukocytes as taking up the majority of the cocci.

However, the original observations of Wyssokowitsch[6] point clearly to the importance of phagocytosis by endothelial cells in the removal of bacteria from the circulating blood. He says: The defensive power of the body lies much more in the structures of the vessel walls; that is, in the endothelial cells of the blood vessels. The bacteria which reach the blood stream are deposited in or between the endothelial cells, which line the capillaries most numerously in organs where

[6] Wyssokowitsch, *Z. Hyg.*, 1886, i, 3.

[7] Kyes, P., *J. Infect. Dis.*, 1916, xviii, 277.

[8] Manwaring, W. H., and Coe, H. C., *J. Immunol.*, 1916, i, 401.

[9] Bartlett, C. J., and Ozaki, Y., *J. Med. Research*, 1916–17, xxx, 465.

the blood stream is slowest. It is here that the battle between the cells and bacteria concerning which we have so much evidence from many sides takes place. However, we still have no decisive knowledge of the course of this conflict, nor of the means of attack and defense.

In sections from the organs of recently injected animals it is difficult to determine how the streptococci are taken up. We have studied chiefly the lung, as this is the organ most concerned in their removal. In lung sections of animals killed 10 to 30 minutes after injection it is clear that some of the cocci are held within the leukocytes in the capillaries. It is equally clear that the majority are not located within leukocytes. Many of them lie in large mononuclear cells which are probably the swollen endothelium of the capillaries; others lie in an eosin-staining matrix which may represent a section through endothelial protoplasm, or possibly some substance from the blood deposited about them.

In order to study this point more accurately, we have etherized and killed cats within 30 minutes after injection, washed the lungs as free as possible by perfusion with salt solution, and then fixed them by injecting Helly's fluid under moderate pressure in order to keep the capillaries distended. In sections fixed in this way the streptococci are not washed from the capillaries; even leukocytes are still present in moderate numbers. Of the mechanism by which the cocci are held, however, we are still in doubt, though many appear to be in endothelial cells.[10]

Fate of the Phagocyted Cocci.

More important for the understanding of the resistance to streptococcic infection than the mechanism of removal of the organisms from the circulation seems to be the means by which they are ultimately destroyed.

The possibility that the body freed itself by excreting the living bacteria was considered by Wyssokowitsch[6] and ruled out on account of the small number which he could recover from the urine, intestinal contents, or milk. Our own observations on the urine and bile of infected rabbits made clear that few living streptococci were passed

[10] We wish to acknowledge our indebtedness to Prof. W. G. MacCallum for his advice and cooperation in these experiments.

out in these secretions. It seemed then that there must be within the body some mechanism for their destruction. In the rabbit this proc ess is obscured by the fact that after a period of apparent destruction the bacteria in the blood stream again begin to multiply. The cat, however, is practically insusceptible to artificial streptococcus infection and large numbers of organisms introduced into the blood are promptly removed as in the rabbit and do not again reappear, or at least reappear only for a brief period.

It has been seen that the streptococci which disappear from the blood are taken up in part by the leukocytes but probably more extensively by the endothelial cells, at least the majority are held in the viscera by some mechanism other than ingestion by polynuclears. The fate of the organisms taken up in these two ways was studied, as far as possible separately.

Bactericidal Effect of Leukocytes.

There is no doubt, as stated above, that a considerable number of cocci is taken up by the leukocytes, but the fate of the organisms so taken up is by no means clear. Schattenfroh,[11] Pettersson,[12] Kling,[13] Zinsser,[14] and others have shown that extracts of leukocytes have distinct bactericidal action on many species of organisms, although Watabiki[15] has failed to demonstrate this action. However, when whole living leukocytes have been tested, Pettersson, Kling, and most others who have investigated their action have failed to demonstrate bactericidal effect.

Our results have not been entirely clean-cut. We have used both whole clotted blood and cells from citrated blood. The technique for clotted blood was as follows: An animal was bled shortly after injection and 1 cc. of blood was immediately measured into each of a series of test-tubes. One sample was emptied into a mortar as soon as clotted, ground with sand, and poured into melted agar. The test-tube and mortar were rinsed with saline solution, the wash was added

[11] Schattenfroh, A., *Arch. Hyg.*, 1897, xxxi, 1.
[12] Pettersson, A., *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 498.
[13] Kling, C. A., *Z. Immunitätsforsch., Orig.*, 1910, vii, 1.
[14] Zinsser, H., *J. Med. Research*, 1910, xxii, 397.
[15] Watabiki, T., *J. Infect. Dis.*, 1909, vi, 319.

to the agar, and the whole plated. Other tubes were plated in this way after various periods of incubation. In one instance there appeared to be a distinct reduction in the blood of a normal cat (Table III). In normal or immune rabbits there was a slight reduction or a gradual increase. The method is not sufficiently accurate to enable one to attach significance to these irregular results. It is impossible to obtain separate colonies from all the streptococci from clotted blood, as is shown by the fact that the count from blood ground immediately after coagulation is much lower than that before coagulation.

On account of the unreliability of this method we made other experiments in which the animal was bled into citrate solution, and the cells were thrown down in the centrifuge, washed once in saline solution, and resuspended in serum or in saline solution. They were then inoculated with a small amount of streptococcus suspension and the measured amount was plated at various intervals. The result of an experiment with normal cat cells and serum is shown in Experiment 6.

Experiment 6.—0.1 cc. of 1.25 cc. of cells plus 1.25 cc. of serum showed 550 colonies immediately, 520 after 1 hour, and 609, 750, and 2,700 after 2, 4, and 8 hours respectively. 0.1 cc. of 1.25 cc. of cells plus 1.25 cc. of salt solution gave 500 colonies immediately; 400, 450, 600, and 3,000 after 1, 2, 4, and 8 hours respectively. 0.1 cc. of 2.5 cc. of serum alone gave 280 colonies immediately; 300, 170, 170, and 3,000 after 1, 2, 4, and 8 hours respectively. 0.1 cc. of 2.5 cc. of salt solution alone gave 42 colonies immediately; 0, 0, 1, and 0 colonies after 1, 2, 4, and 8 hours respectively.

Control tubes of blood cells in serum and blood cells in salt solution were inoculated with a large number of streptococci for morphological study. Films made after 1 hour's incubation showed extensive phagocytosis both in serum and salt solution, but more marked in the serum tubes.

The only tube in which there was a distinct destruction of the streptococci was in the salt solution control. Other experiments in which normal or immune rabbit serum and cells were used gave similar results. In the tests made with citrated cells we never observed a reduction in the count comparable to the isolated result with clotted blood recorded in Table III. We are uncertain whether or not the slight reduction in the counts from the serum tube in the experiment

above, or similar reductions in tubes of serum and cells in other experiments indicate a feeble bactericidal action on the part of these elements. It seems certain, however, that if it exists it is much inferior to the destructive power of the lung tissue which we are about to discuss, and that it is entirely insufficient to account for the extensive destruction of the injected bacteria which takes place. It seems unlikely from these experiments that the leukocytes can even destroy the small shares of these organisms which they can be seen to ingest.

Bactericidal Effect of the Tissues.

In contrast to this doubtful evidence that the leukocytes or serum can take part in the destruction of invading streptococci, it can be clearly shown that certain tissues, especially the lung, are possessed of marked bactericidal power. The most convincing evidence is that obtained from cats killed at various intervals after injection.

If a cat is killed within a half hour after receiving streptococci intravenously, cultures show an enormous number of the cocci in the lungs, many in the liver and spleen, and in the muscles, blood, and kidneys a few or none at all, the result depending on the number injected (Tables II and IV). In a few experiments in which the bone marrow and lymph nodes were also examined these tissues contained far less than the spleen but rather more than the last mentioned tissues.

Cultures from a cat killed several hours after an injection show a smaller number of colonies from all the viscera, but perhaps a slightly increased number in the muscles. The decrease is most marked in the lung, but is also pronounced in the liver. The spleen seems to rid itself more slowly of the cocci. If the dose is small, all the tissues may be found sterile in 8 hours; if very large, positive cultures may be obtained for more than 24 hours. In the cats recorded in Table II the tissues were practically sterile in 24 hours. The most interesting point is that when films were made from the ground lung tissue at a stage when the cultures showed a greatly diminished number of viable bacteria, or none at all, numerous stainable streptococci were found. They were found up to the 5th day, which is as far as our observations have extended. In comparing Cats 1 and 4 it is seen

that, whereas in 5 days the viable bacteria had dropped from 300,000 to 0 per dg., the number of organisms seen in films seemed undiminished. This seems to admit of but one interpretation; namely, that the streptococci have been taken up in the lungs and killed, although they remained visible for a considerable period of time.

Fate of Streptococci in Cat Tissues.—Four cats were injected intravenously with 40 million streptococci per kilo and killed at the intervals noted. Pieces of the organs to be studied were placed in a sterile test-tube, weighed, ground in a sterile mortar with sand, and emulsified in salt solution added in the proportion of 1 cc. to 0.1 gm. of tissue. Plates were made in sheep blood agar of amounts of organ emulsion representing from 0.1 to 0.0001 gm. and the colonies counted after 24 hours. Films were made from the ground emulsion and stained by Gram's method. As previous experiments had shown most pronounced effect in the lung, the lung cultures were made in duplicate from different parts of the organ.

TABLE II.

Tissue.	Cat 1. Killed 10 min. after injection.			Cat 2. Killed 5 hrs. after injection.		Cat 3. Killed 24 hrs. after injection.		Cat 4. Killed 5 days after injection.	
	Colonies per 0.1 gm.								
	Film.	Tissue ground and plated immediately.	Tissue ground and plated after 5 hrs. incubation.	Film.	Colonies per 0.1 gm.	Film.	Colonies per 0.1 gm.	Film.	Colonies per 0.1 gm.
Lung	−−−	300,000	160,000	++	800	+++	1	+++	0
		330,000	80,000		72		0		0
Spleen	+	18,000	140,000	0	1,000	0	1	0	0
Liver	+	34,000	44,000	0	43	0	0	0	0
Kidney		100	3,000*		3		0		0
Bone marrow	0	500			7		2		0
Psoas	0	8	52	0	20	0	0		0
Pectoralis		27	About 12,000		53		0		0
Blood		26	1,200		12		0		0

* 3,000 indicates that the colonies in a plate from 0.1 gm. were too numerous to estimate and that no plates were made from smaller amounts of tissue.

It seemed important to attempt to demonstrate this bactericidal action of tissue, which is so pronounced in the lung *in vitro*. In the

experiment in Table II pieces of the organs from the cat killed 10 minutes after injection were removed and distributed in sterile test-tubes which were placed in a moist chamber at 37°C. for 5 hours, in order to compare the action of the excised tissue with that of the tissue of the cat which had been allowed to live for the same period. At the end of this time the pieces were weighed, ground, and measured amounts planted as shown in Table II. It will be noted that in the living animal there was an enormous reduction in the number of organisms in the lung and liver and a less pronounced reduction in the spleen, bone marrow, and blood. The reduction in the last may well have been due to other factors than the bactericidal effect of the blood itself. On the other hand, there was an increase in the number of bacteria in the muscles during this period.

If these findings are compared with the fate of the bacteria in Cat 1 which was killed 10 minutes after injection, it will be noted that the lungs showed a reduction in the number of bacteria which was distinct though scarcely comparable with that taking place in the living

TABLE III.

Bactericidal Effect of Cat Tissues.

A normal cat weighing about 3 kilos was injected with 2 cc. of a streptococcus suspension containing 30 million per cc. In 10 minutes it was bled to death under ether and perfused for 10 minutes through the jugular to wash the blood from the lung. Pieces of the lung were placed in sterile test-tubes, and the animal was then perfused 10 minutes through the proximal portion of the carotid to remove the blood from the other tissues. Pieces of the other tissues were then distributed in sterile test-tubes. They were incubated, weighed, ground, and plated as in the preceding experiment.

Tissue.	Colonies per 0.1 gm. of tissue.				
	Plated immediately.	After 1 hr.'s incubation.	After 2 hrs.' incubation.	After 4 hrs.' incubation.	After 11 hrs.' incubation.
Lung	26,000	18,000	3,300	9,000	2,400
	26,000		750	2,000	300,000
Spleen	360		1,000	3,700	3,000
Liver	1,300		560	920	3,000
Kidney	0		8	32	54,000
Psoas	1		—	0	1,500
Blood	20		0.3	9.2	12

animal, whereas in the spleen, liver, and blood the organisms multiplied moderately, and in the kidney and muscle tissues multiplied with great speed. Further experiments showed that the destruction by excised tissue took place only during the first 2 or 3 hours of incubation, after which, owing probably to death of the cells, they again multiplied. This is illustrated in Table III.

TABLE IV.

Bactericidal Test of Cat and Rabbit Tissues.

A normal cat weighing 4,000 gm. was injected intravenously with 4 cc. of streptococcus suspension containing 400,000 per cc. (400,000 per kilo). It was killed with gas in 10 minutes, and the specimen removed and cultures were made as before.

A rabbit weighing 1,600 gm. was given 3.2 cc. of suspension containing 200,000 per cc. (400,000 per kilo). It was killed in 10 minutes and cultures were made as above.

Tissue.	Plated immediately.	After 1 hr.'s incubation.	After 2 hrs.' incubation.	After 3 hrs.' incubation.	After 4 hrs.' incubation.	After 6 hrs.' incubation.	After 10 hrs.' incubation.
Cat.							
Lung 0.1 gm.	2,600	2,800	100	140	1,600	4,000	∝
" 0.1 "	3,700	2,100	720	1,200	105	5,200	
Spleen 0.1 "	6		2				∝
Psoas 0.1 "	0		0				0
Blood 1 cc.	0						
Rabbit.							
Lung 0.1 gm.	750	720	710	3,000	19,000	∝	∝
" 0.1 "	1,400	750	1,200	4,500	17,000	∝	
Spleen 0.1 "	70		900				
Psoas 0.1 "	0		0				
Blood 1 cc.	0						

This experiment seems to show a definite decrease in the number of viable bacteria in the lung tissue when incubated. The action of the tissue was evidently much impaired by its removal from the body and after a few hours the bacteria multiplied freely in the dead tissue. The decrease in the lung is more striking if compared with the prompt increase in the spleen, or, in experiments with rabbits, in the muscles.

The process can be more definitely demonstrated if a much smaller injection is made; in fact it is difficult to compare the organs of the same animals *in vitro*, as, if the injection is small enough to show clearly the effect in the lung, there are not sufficient bacteria deposited in the other organs to give positive cultures from amounts which it is practicable to test.

Table IV records the results observed in a cat which was given a much smaller injection, so that the blood was completely sterilized in 10 minutes. In this experiment a rabbit was injected for comparison with a proportional dose of the same suspension. The difference in the distribution of the organisms and the effect of the tissues on them will be referred to later.

From the results recorded in the preceding experiments there seemed little doubt as to the killing of the streptococci in the lung. However, on account of the large differences between duplicate samples of lung tissue, during the later stages of these experiments it seemed best to repeat the experiment in quadruple. The results are recorded in Experiment 7, in which each entry represents the count from a piece of lung weighed, ground, and plated separately.

Experiment 7. Bactericidal Effect of Lung Tissue of Cat.—A normal cat was inoculated with 12 cc. of a streptococcus suspension containing 100 million per cc. The animal was killed with gas after 10 minutes. The autopsy was completed and first plants were made 30 minutes after injection.

0.1 gm. of lung tissue showed 550, 248, 200, and 480 colonies immediately; 34, 40, 28, and 60 colonies after 2 hours; and 120, 53, 25, and 65 colonies after 5 hours.

0.1 gm. of spleen tissue showed 146 colonies immediately; 194 after 2 hours; and 3,000 after 5 hours.

The reduction in the number of colonies obtained from the lung after incubation was so constant and so marked that, even though it varied in degree in different pieces of the same lung, it seemed certain that it was due to an extensive destruction of the streptococci which took place in the tissue *in vitro*. The question at once arose as to what element of the tissue was responsible for this destruction. The uncertainty as to the location of the cocci in the lung makes this difficult to answer. If, as appears from a study of the sections, the majority of the cocci are taken up by endothelium, it is most likely that these are the cells which destroy them. That the bactericidal

power lies in some of the fixed tissue elements and not in the blood appears certain from the following considerations: In the first place, the experiments described above show that the whole blood, the blood cells, and the blood serum are incapable of destroying any considerable number of streptococci. In the second place, the tests recorded in Table III were made with tissue that had been freed as much as possible from blood and from free lying cocci by perfusion before the pieces were excised for examination. Moreover, in order to rule out participation of the elements of the blood in the taking up and destruction of the streptococci we performed the following experiment:

Experiment 8.—A cat was first perfused for from 2 to 3 hours with citrate and Ringer's solution, and then perfused with a very dilute streptococcus suspension (about 400 organisms per cc.). The lung was again perfused with sterile Ringer's solution until the content of the outflow was reduced to 35 per cc. Pieces of lung were then removed and treated as in the previous experiments.

Culture from 0.1 gm. of perfused lung gave 250 and 170 colonies immediately; 42 and 3 after 2 hours; 16 and 0 after 4 hours; 0 colonies after 7 and 24 hours.

In view of the fact previously mentioned, that streptococci quickly die when placed in a dilute suspension in salt solution, the possibility must be admitted that in this instance the Ringer's solution took part in their destruction. However, it has been shown that the blood has no marked bactericidal power, but that the lung with the blood still in the capillaries (Experiment 7), the lung perfused with Ringer's solution after the streptococci had been taken up (Table III), and the lung perfused before the streptococci were injected (Experiment 8), all show a marked bactericidal effect; and the conclusion is forced upon one that this power lies in the lung tissue itself.

Incidentally the last experiment mentioned raises a doubt as to the importance of the endothelial opsonin of Manwaring in this process, as the streptococci were taken up and destroyed by a lung which had been previously perfused for over 2 hours in order to free it of blood.

In fact it seems doubtful if humoral protective substances are active here. In one experiment in which a cat that had received streptococci sensitized with immune rabbit serum was tested with another which was given unsensitized bacteria there was little difference

between the two animals; in fact the destruction of the unsensitized cocci was the more marked. Experiments with immune rabbits, which we shall refer to later, indicate that immunization may influence the taking up of the cocci by the tissues, but does not affect the manner or extent of their destruction.

This bactericidal action of the lung tissue which can be demonstrated *in vitro* must doubtless be due to some enzyme or antibody-like substance and we have made repeated attempts to demonstrate some such destructive action in extracts of fresh lung tissue. Portions of lung of freshly killed cats were removed and ground with sand, in fresh cat serum, and separated by brief centrifugation into an opalescent supernatant fluid and a muddy sediment. Both supernatant fluid and sediment were tested by inoculating them with a small amount of streptococcus culture, and plating measured amounts after various periods of incubation. Experiments carried out in this way have failed to show any definite bactericidal effect, though a slight reduction in the number of colonies may appear, as shown in Experiment 9. However, a control tube of the same extract previously heated at 56°C. for 20 minutes showed almost as marked a drop.

Experiment 9.—Lung extract unheated gave 900 colonies immediately; after 1, 2, 4, and 8 hours it gave 800, 600, 2,700 and 3,000 respectively.

Lung extract heated gave 1,000 colonies immediately; after 1, 2, 4, and 8 hours it gave 1,200, 750, 2,000, and 3,000 respectively.

Ground lung tissue gave 150 colonies immediately; after 1, 2, 4, and 8 hours, 450, 750, 1,200, and 3,000 colonies respectively.

It is evident then that streptococci withdrawn from the circulation of normal cats by the lung are promptly killed in the living animal and that pieces of excised lung carry out this same process in the test-tube. The bactericidal power seems to depend, however, on the living conditions of the cells, is much enfeebled by the removal of the tissue from the body, and is not exhibited at all by fresh extracts of the tissue.

With regard to the other organs there seems little doubt that in the living animal the liver destroys many of the organisms taken up, and in some experiments, Table III, for example, this destruction apparently took place *in vitro*.

The disappearance from the blood we believe, for reasons already given, to be due to a taking up and destruction of the bacteria by the viscera and not to any bactericidal power in the blood plasma or cells. The spleen, and in a few experiments the muscle, kidney, and bone marrow, have failed to show bactericidal power *in vitro* and it is impossible to determine from our data whether the disappearance of the streptococci from these organs in the living animal is due to a destruction *in vivo* or to their being carried by the blood to the lung or liver and destroyed there.

Comparison between Susceptible and Resistant Animals.

Our observations on cats have been presented in detail in order to make clear the evidence on which we based our conclusions as to the fate of the injected streptococci. The significance, for the protection of the animal, of this mechanism for taking up and destroying the injected cocci becomes more apparent if we compare the findings in cats which are resistant to the infection with those in rabbits which are susceptible. These we shall present briefly.

The ability of rabbits to free their circulation temporarily of injected streptococci has already been discussed. The subsequent course of the injection is illustrated in Table V.

The first difference between the rabbits and the cats is in the primary distribution of the invading bacteria. In the experiments in Table V about equal amounts were deposited in the lung, liver, and spleen, and in other experiments the number taken by liver and spleen considerably exceeded that taken by the lung (Table VI), whereas in the cats, the lungs as a rule take up several times as many as these other organs. In the rabbits, too, a detectable number was taken up by the muscles and many by the bone marrow.

The findings in the lung of the rabbit killed after 8 hours are similar to the condition in cats killed at the corresponding period, in that the culture of the lung showed a greatly diminished number of streptococci, although they were still found without difficulty in film preparations. At this stage the liver and spleen also appear to have killed most of the organisms, which they have taken up. As in the cats, a feeble bactericidal action by lung tissue could be demonstrated

TABLE V.

Fate of Streptococci in Rabbit Tissues.

Three normal rabbits were injected with a suspension of streptococci, the doses being about 160,000,000 per kilo.

Rabbit 6 was killed in 10 min.

Rabbit 7 was killed after 8 hrs.

Rabbit 8 was found moribund on the 4th day and was killed.

Portions of the organs were weighed, ground with sand, and plated in amounts representing from 0.1 to 0.001 gm. of tissue. The results are recorded as the number of colonies obtained from 0.1 gm. of tissue.

Tissue.	Rabbit 6 (10 min.)		Rabbit 7 (8 hrs.)	Rabbit 8 (4 days)
	Film.	Colonies.	Colonies.	Colonies.
Lung	++	54,000	220	18,000
"		76,000	220	19,000
Spleen	+	34,000	230	5,000
Liver	++	116,000	0	44,000
Kidney		0	0	127,000
Psoas		75	116	98,000
Quadriceps			750	61,000
Heart muscle				80,000
Bone marrow		5,000	0	21,000
Axillary lymph node				59,000
Mesenteric lymph node				190,000
Blood (1 cc.)		1,200	42	6,600

in vitro, whereas streptococci multiply with great rapidity in spleen and muscle tissue removed from the body.

As far as one can determine from the experiments *in vitro* the bactericidal power of lung tissue of the rabbit is distinctly inferior to that of the cat, as is shown in Table IV, where the tissues of the two species were tested after the animals had been given proportional injections. In other cases, however, for example Table VI, rabbit lung tissue showed distinct bactericidal power *in vitro* and the observations on living animals indicate (Table V) that the organisms taken up by a rabbit's lung and liver are largely disposed of. The more striking difference between the susceptible and insusceptible animals is that in the rabbit a considerable number of bacteria lodged in the muscle tissues and sometimes in the kidneys, and that these multi-

plied slowly in the living animal and with remarkable rapidity in the excised tissue.

Just how these organisms are deposited in the muscle tissue of the rabbit we have not yet determined, and in fact a study of this point is difficult because the number primarily taken up is so small that the bacteria are difficult to find in sections. The fact, however, that in this tissue the streptococci are removed from the blood stream and nevertheless proceed to grow, whereas in the lung and liver they are

TABLE VI.

Bactericidal Effect of Rabbit Tissues.

A normal rabbit was injected under ether intravenously with a small dose of streptococci and bled to death in 10 minutes. The lungs were perfused briefly with Ringer's solution and portions placed in test-tubes. The other organs were then perfused and specimens similarly taken. Samples of blood were distributed in 1 cc. amounts and allowed to clot. Pieces of tissue were weighed, ground, and plated at intervals as in previous experiments.

Tissue.	Colonies per 0.1 gm. of tissue tested.				
	Plated immediately.	After 1 hr.'s incubation.	After 2 hrs.' incubation.	After 4 hrs.' incubation.	After 8 hrs.' incubation.
Lung	8,800	1,600	800	1,500	32,000
"	10,000		3,300	3,200	24,000
Spleen	75,000		60,000	360,000	300,000
Liver	24,000		46,000	100,000	300,000
Kidney	140		200	1,500	3,000
Psoas	6		11	110	3,000
Blood	180		100	130	750

removed from the blood stream and killed, forces the conclusion that there must be an essential difference in the way they are held by the muscle tissues to account for their unrestricted multiplication.

Our observations seem to indicate that the susceptibility of the rabbit to the infection is determined not by the inability of the lung and other tissues to kill the streptococci which they take up primarily, but by the fact that a considerable number of the organisms lodge in the muscles and other tissues which lack this bactericidal power and that they multiply almost from the first in these tissues where they are found in enormous numbers at the time of death. The

great number found at the time of death in the lung and liver, organs which were able to free themselves almost entirely of living cocci during the first period of the infection, may well indicate that toward the termination of the infection these resistant organs are overwhelmed with bacteria brought to them from the muscles where they have been growing without hindrance.

Wyssokowitsch[6] considered this possibility. He said:[16] From the experiments so far performed, one cannot determine with certainty whether also in this case (a susceptible animal) the bacteria taken up by certain organs are not destroyed, and whether perhaps only in certain locations the bacteria survive and then multiply. One might suppose that the body is gradually overwhelmed with newly developed bacteria from the least resistant tissue.

Fate of Streptococci in Immune Rabbits.

The next point which we desired to investigate was the effect of active or passive immunization on the rabbit's ability to rid itself of the invading organisms. Our observations on this point are incomplete and unsatisfactory, chiefly on account of the difficulty in producing in rabbits a reliable immunity to the streptococcus. We have treated rabbits for as long as 8 months with intravenous injections of killed streptococci and found that when they were injected with an amount sufficient to kill a normal animal in 2 or 3 days, they usually succumbed. Our investigations are not numerous enough to lay down general rules as to the course of these infections, but it appears that rabbits so immunized live somewhat longer than normal controls, and that after 12 to 24 hours their blood stream becomes free of streptococci. Later they develop a local infection, frequently in the joints, and subsequently succumb to septicemia.

This occurrence of local infection in immunized or possibly sensitized animals opens a field for further study. It would seem to have a direct bearing on the problem of local infections in man. The observations of Wadsworth[17] on pneumonia in rabbits, and of Faber[18] on streptococcus arthritis at once come to mind in this connection.

[16] Wyssokowitsch,[6] p. 40.
[17] Wadsworth, A., *Am. J. Med. Sc.*, 1904, cxxvii, 851.
[18] Faber, H. K., *J. Exp. Med.*, 1915, xxii, 615.

An experiment in which the reactions of an immunized and of a normal rabbit to injections of streptococci were compared is recorded in Table VII.

TABLE VII.

Comparison between Normal and Immunized Rabbits.

A normal rabbit and a rabbit which had been treated 2 months with intravenous injections of killed Streptococcus 43 were injected into the left ventricle with 8 cc. per kilo of a suspension of the same streptococcus containing 20,000,000 per cc. They were bled to death under ether 10 minutes after being injected, and the organs treated as in previous experiments.

Tissue.	Colonies per 0.1 gm. of tissue.			
	Immune rabbit.		Normal rabbit.	
	Plated immediately.	After 4 hrs.' incubation.	Plated immediately.	After 4 hrs.' incubation.
Lung	170,000	119,000	61,000	—
"	173,000	128,000	21,000	—
Spleen	4,000	138,000	120,000	1,800,000
Liver	10,000	2,000,000	104,000	6,000,000
Kidney	0	0	0	0
Psoas	31	1,000	1,500	3,000
Quadriceps	130	1,200	2,400	3,000
Blood (1 cc. clotted)	1,000	3,000	—	—

In the brief period of observation the removal of the cocci from the circulation of the immunized rabbit was no more complete, in fact even less so than from that of the normal. Cultures from the carotid 10 minutes after injection showed 35,000 in the immune and 21,000 in the normal animal.

In this instance the lung tissue of the immune rabbit showed a questionable bactericidal power, but at least there was inhibition of growth by the lung as compared with rapid multiplication of the cocci in the other tissues. Similar results have sometimes been obtained with normal rabbit tissues (Table IV). The only marked difference between the immune and the normal animal is in the primary distribution of the cocci. It will be noted that while in the normal rabbit (Tables V and VI) the spleen and liver take up nearly as many or more cocci than the lung, in the immunized rabbit the lung has seized 17 times as many as the liver and 40 times as many as the spleen. In other words, the ratio of distribution of the cocci among

the organs of the immunized rabbit is roughly that found in normal cats.

On account of unavoidable interruption of our work we have been unable to extend further these preliminary observations on the effect of immunization.

CONCLUSIONS.

1. Streptococci injected into the circulation of cats are quickly withdrawn and are found most numerously in the lung, less numerously in the liver and spleen, and in small numbers in the bone marrow, lymph nodes, muscle, and kidney.

2. The streptococci taken up by the lung are killed within 5 to 8 hours, although they remain visible in films for a number of days. In the liver they are killed less rapidly, and in the spleen a few may remain viable for a considerable period.

3. This bactericidal action may be demonstrated in pieces of excised lung but not in lung extracts, and is apparently dependent on the action of the living cell.

4. Streptococci injected into a susceptible animal, the rabbit, are also promptly removed from the circulation, but are distributed in different proportions, the liver and spleen absorbing almost as many as the lung, and the muscles also taking up an appreciable number.

5. As in the cat, the organisms taken up by the lung and liver of the living rabbit are promptly killed. Those which lodge in the muscles, however, multiply rapidly.

6. About the time that the streptococci have begun to develop in the muscles (4 to 8 hours after injection) the number in the blood stream begins to increase.

7. The increase in the blood stream is not due to exhaustion of the mechanism of their removal nor have these organisms acquired a resistance sufficient to maintain them in the blood stream of a normal animal. The septicemia, then, is probably the result of washing out of organisms from the infected tissues.

8. Attempts to immunize rabbits have been unsuccessful, but in certain treated animals the distribution of the organisms among the various organs approached that found in insusceptible animals; *i.e.*, cats.