A METHOD FOR ESTIMATING THE BACTERIA IN THE CIRCULATING BLOOD IN RABBITS.*

By CARROLL G. BULL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Not a few of the determinations in experimental bacteriology have to do with the testing of bacteria for their infectious power. The laboratory animals principally employed for this purpose are small, and afford few reliable criteria which can be used to decide the result. The criterion which, because of its very definite character, is usually relied upon is the development of severe illness and the intervention of death; but this criterion cannot take account of degrees of intensity of infection, except as they affect the period of survival of the animal after inoculation, which is manifestly a coarse differential. All who have conducted so called virulence tests with bacteria have felt the need of other means of differentiation than mere survival or death of the inoculated animal. In the course of some experiments on the therapeutics of streptococcal and pneumococcal infections, this need became imperative, so that a means was sought that should yield data capable of comparison at different periods and intervals after inoculation, and which did not rely alone on the ultimate result of recovery or death.

The starting point of the quest was the well known phenomenon, first observed by von Fodor[1] and investigated accurately by Wyssokowitsch,[2] namely, that bacteria injected into the circulation are soon filtered out of the blood and, according to their degree of infectiousness, are destroyed or reappear, multiply, and, when of sufficient power, ultimately cause death. Hence the question which arose was whether the disappearance, reappearance, and multiplication proceed with a degree of regularity and consistency rendering a numerical estimate feasible of the bacteria in the circulating blood.

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[1] von Fodor, J., *Arch. f. Hyg.*, 1886, iv, 130.

[2] Wyssokowitsch, W., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1886. i. 3.

The later studies of Weil[3] and of Reichstein,[4] which deal with the fate and the estimation of streptococci within the blood stream, offered an encouraging affirmative on this point, so that the next question to arise was whether blood taken repeatedly from superficial vessels sufficed for the purposes of the estimation.

EXPERIMENTAL PART.

Bacteria and Injections.—The streptococcus used in the following experiments was originally isolated from the throat of a scarlet fever patient. It is a typical hemolytic, Gram-positive, chain-forming streptococcus. It has been repeatedly passed through rabbits and, at the beginning of this work, 0.25 of a cubic centimeter of a twenty-four-hour bouillon culture[5] per kilo of body-weight killed rabbits within one to five days. The pneumococcus used belongs to group I according to Neufeld's classification. Its virulence has been maintained by repeated passages through mice and rabbits. 0.001 of a cubic centimeter of a twenty-four-hour bouillon culture per kilo of body-weight kills rabbits within one to three days. In all experiments the bacteria were injected into the ear veins.

Taking the Blood.—The uninjected ear was carefully shaved and washed with 95 per cent. alcohol. The marginal vein was pricked and a few drops of blood allowed to flow out before any was taken for the cultures. Then a definite number of drops were caught directly into sterile Petri dishes. A tube of agar, previously melted and cooled to 42° C., was immediately poured into the dishes and the blood and agar were thoroughly mixed. Two or more plates

[3] Weil, E., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1911, lxviii, 346.

[4] Reichstein, S., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1913, lxxiii, 209.

[5] We have found blood bouillon (3 to 5 drops of sterile defibrinated rabbit blood in a tube of 5 c.c. of beef infusion bouillon) to be an excellent medium for streptococci and pneumococci. The difficulty of "infecting" large quantities of medium with loop inoculations from bouillon to bouillon is eliminated (Gillespie). The bacteria do not lose this virulence as readily in this medium as in ordinary bouillon. Another advantage is that the bacteria live in this medium for long periods of time without being transferred. Streptococci will live for 6 months when the cultures are kept in the dark at low temperature; it is not necessary to seal the tubes in any way. Pneumococci will live for at least 6 weeks. This medium was used throughout the experiments instead of plain bouillon.

were made from each rabbit, the amount of blood varying from one to ten drops, according to the supposed degree of infection. In this way plates were obtained on which the colonies could be easily counted and the various plates compared.

The above procedure permits of frequent taking of blood with the least possible disturbance to the rabbit. Contaminations rarely occur if the ear is properly cleansed and a few drops are allowed to flow out before taking the blood for the cultures. Blood from the first few drops adheres to the surface of the ear and the following drops run over this without coming in contact with the skin. The accuracy of the method might be objected to, because the measuring of the blood is as inaccurate as the size of one drop of blood may differ from another. If the veins are pricked by a stab with the same sharp-pointed instrument and the surface of the ear is held in a perpendicular position so as not to allow the blood to collect in large quantities before flowing off, drops of fairly constant size can be obtained and the number of colonies on various plates of the same number of drops is remarkably uniform. The accuracy of the procedure can also be tested by infecting a series of rabbits with the same quantity of a bacterial suspension per kilo of body-weight and taking cultures at stated intervals in a short time after the injections. Experiment 1, as given below, was done with this object in view.

STREPTOCOCCUS INFECTIONS.

Experiment 1.—Each of seven rabbits was injected intravenously with 0.25 c.c. of a fresh bouillon culture of streptococci per kilo of body-weight. Cultures were made from the opposite ear 30 minutes and 5½ hours afterwards. In this, as in all other experiments, the number of colonies was calculated on the basis of ten drops of blood. The results are given in table I.

TABLE I.

	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.	Rabbit 5.	Rabbit 6.	Rabbit 7.
Colonies at 30 min.....	140	145	135	100	130	130	100
Colonies at 5½ hrs.....	8	9	20	22	10	12	10

Many experiments of this character were performed and similar results were always obtained. The first cultures were made after

ample time had elapsed for a thorough and uniform distribution of the bacteria in the blood stream and the number of colonies obtained from the different rabbits shows only slight variations. At the next bleeding, five hours later, the plates were still uniform, although a great reduction in the number of colonies had occurred. These results are probably due to the fact that this initial disappearance of the bacteria from the blood depends largely upon mechanical forces which are about the same in each animal. A biological reaction between the host and the infecting organism had evidently played a very slight rôle up to this time; otherwise, such uniform results could not be expected. Such an experiment also shows that this method of taking blood gives dependable data.

In following the progress of the infections further, it was found that they may take one of three courses, mainly depending upon the virulence of the bacteria. A series of experiments was carried out just after the bacteria had been passed through a number of rabbits. The infections ran an acute course, the blood was never free of bacteria, and the autopsies showed no evidence of localization. After two months' cultivation on artificial media, no animal passages being made, the same amount of bacteria caused a chronic infection in a majority of the animals. There were longer or shorter intervals during which the septicemias were slight or entirely absent. After a time the bacteria suddenly reappeared in the blood and the animals died within a few hours to two days. Autopsies showed localized infections, from which the second blood invasion undoubtedly came. When the bacteria were still less virulent, they permanently disappeared from the blood and the animals recovered. Examples of these types of infection are reported in the following tables.

Experiment 2.—Each rabbit was given 0.25 c.c. per kilo of body-weight of the ame suspension of streptococci into the ear vein. The blood for the cultures was taken from the opposite ear at the times indicated in the protocols. The number of colonies was estimated on the basis of ten drops of blood.

An examination of table II shows that the infections, with the exception of rabbits 2 and 3, ran a remarkably uniform course. From the time of the injections to about five hours afterwards there is a rapid decrease in the number of bacteria. From five to six

TABLE II.[6]

Animal.	Time of bleeding and number of colonies.											
Rabbit 1,	30 min.	2 hrs.	6 hrs.	10 hrs.	22 hrs.	48 hrs.	72 hrs.	Died 1 hr. after the last bleeding.				
weight 1,800 gm.	52	1	18	12	9	100	×					
Rabbit 2,	30 min.	2 hrs.	6 hrs.	11 hrs.	23 hrs.	30 hrs.	47 hrs.	56 hrs.	Was dead at 64 hrs.			
weight 2,000 gm.	200	5	100	200	1000	×	×	950				
Rabbit 3,	30 min.	2 hrs.	6 hrs.	10 hrs.	22 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	126 hrs.	138 hrs.	Dead at 144 hrs.
weight 1,600 gm.	110	9	13	35	65	1500	×	100	75	×	100	
Rabbit 4,	30 min.	6 hrs.	18 hrs.	Dead at 36 hrs.								
weight 1,500 gm.	25	100	2000									
Rabbit 5,	30 min.	2 hrs.	6 hrs.	11 hrs.	23 hrs.	30 hrs.	36 hrs.	Dead at 38 hrs.				
weight 1,750 gm.	75	5	70	245	×	×	×					
Rabbit 6,	30 min.	3 hrs.	18 hrs.	24 hrs.	42 hrs.	48 hrs.	Dead at 50 hrs.					
weight 1,100 gm.	50	6	1000	2000	×	×						

[6] In the tables, × indicates that the plates were so heavy that the colonies could not be counted.

hours afterwards the bacteria begin to increase in number and septicemia becomes heavier and heavier until the death of the animal. The rabbits died in from one and a half to three days. Rabbits 2 and 3, especially 3, showed some variations from this rapid, progressive course. Rabbit 3 lived six days and its blood cultures give a possible explanation for this. At seventy-two hours the blood had reached so great a septicemic state that the animal should have succumbed on the following day. But the rabbit lived and the blood culture showed a retrogression of the infection for two days. This was followed by another exacerbation which was also overcome and succeeded by another retrogression. The rabbit died on the sixth day with a low culture; but six hours elapsed between the last bleeding and the death of the animal. This gave sufficient time for another ascension, which probably occurred, and as a result of which the rabbit died. Such fluctuations or crises cannot be ascribed to the technique, since they were often observed, and also a temporary reduction in the number of bacteria was always accompanied by a prolongation of the rabbit's life. Infections running such zigzag courses are positive indications that a two-sided warfare occurs within the body of the host; the bacteria make advances which are successfully met by the defensive power of the rabbit, but, not being entirely killed out, they make another trial. Such a struggle may be prolonged for several days, and the final results depend upon the relative strengths of the aggressive forces of the invading bacteria and the defensive resources of the infected animal.

Experiment 3.—This experiment was carried out two months after experiment 1. The same strain of streptococcus was used in this experiment and the same quantities of bouillon culture were injected. This strain had been kept on artificial media continuously and had evidently suffered a loss of virulence. The technique was the same as in experiment 1. Two typical rabbits are reported in table III.

The courses of the infections reported in table III show that the bacteria had lost in aggressiveness but they still possessed an insusceptibility to the destructive influences of the host and were yet able to carry on a local fight in some more or less inaccessible place. The local lesions probably lowered the vitality of the animals to

TABLE III.

Animal.	Time of bleeding and number of colonies.
Rabbit 1, weight 1,540 gm. Autopsy.	30 min. 2 hrs. 6 hrs. 24 hrs. 48 hrs. 72 hrs. 96 hrs. 120 hrs. 140 hrs. 144 hrs. 168 hrs. 192 hrs.,
	76 0 0 0 0 0 0 0 0 0 150 rabbit died.
	Pleurisy with effusion. Streptococci abundant in the fluid. Smear from the heart's blood heavily positive.
Rabbit 2, weight 1,720 gm. Autopsy.	30 min. 5 hrs. 12 hrs. 24 hrs. 36 hrs. 48 hrs. 72 hrs. 96 hrs. 120 hrs. 144 hrs. 168 hrs. 192 hrs.
	30 1 80 50 30 30 25 0 10 50 200 ×
	216 hrs., rabbit died. Pericarditis, and extensive cellulitis over the abdomen. Smears from these lesions were heavily loaded with streptococci.

such an extent that the bacteria were able to produce a general infection. Or, on the other hand, the life within the tissues of the host increased the infectivity of the bacteria and they were able to overcome the opposition offered by the rabbit.

Experiment 4.—This experiment was made one month after experiment 3. The bacteria had been kept on artificial media during this time. The quantities of culture used and other points of technique were the same as in the preceding experiments. Three rabbits are given in table IV to represent the nature of infections obtained at this time.

In experiment 4 the bacteria were almost devoid of infecting power and they behaved very much as saprophytes; they rapidly disappeared from the circulation and were not able to reappear. The rabbits showed no signs of disease and remained in perfect condition.

PNEUMOCOCCUS INFECTIONS.

A number of experiments were carried out with the pneumococcus similar to those just described with the streptococcus. Depending upon the virulence and the number of bacteria injected, a pneumococcic infection in the rabbit may take any one of the three courses described in the streptococcic infections. The initial decrease in the number of bacteria in the blood may not be as complete as with the streptococcus, especially if a very large number of bacteria are injected. Pneumococci usually begin to reappear in the blood earlier than streptococci. The infections are more acute and severe. The types of infection are even more defined than with the streptococcus. The acute infections last from two to three days. In from two to three hours the bacteria have largely disappeared from the blood. Cultures taken at five hours usually show an increase in the number of colonies and this reaches a heavy septicemia in from eighteen to twenty-four hours. The animals die within forty-eight to seventy-two hours. Rabbit 1 in table V is an example of this type of infection. If the bacteria have lost in virulence or if a smaller number is injected, a chronic infection occurs. Rabbit 2 of table V represents this class typically. The bacteria disappear from the blood more rapidly and the reappearance is delayed and the increase is slight and is followed by a second decrease. The rabbits usually have a low septicemia for several days. This is followed by a rise and the animal dies within one to two

TABLE IV.

Animal.	Time of bleeding and number of colonies.							
Rabbit 1, weight 1,380 gm.	30 min.	2 hrs.	20 hrs.	26 hrs.	44 hrs.	66 hrs.	72 hrs.	The bacteria never reappeared in the blood and the rabbit was in perfect condition 1 mo. later.
	75	4	3	5	20	0	0	
Rabbit 2, weight 1,620 gm.	10 min.	4 hrs.	20 hrs.	The blood remained sterile and the rabbit was in good condition after 1 mo.				
	180	0	0					
Rabbit 3, weight 1,200 gm.	3 hrs.	20 hrs.	44 hrs.	64 hrs.	112 hrs.	Bacteria never reappeared in the blood, and the rabbit continued well.		
	7	0	12	0	0			

days. At autopsy severe local lesions are always found. The localization may be in the pericardium, pleura, peritoneum, or in the subcutaneous tissues of the abdomen. In one rabbit localization occurred in the kidneys. Any two or more of these places may be involved in the same animal, but many times only one is affected. The bacteria accumulate in great quantities in these localities; when present, septicemia almost invariably precedes death. In the third type the rabbit masters the situation within a few hours; the bacteria disappear from the blood permanently and the animal continues in perfect condition. Rabbit 3 in table V falls in this class.

SUMMARY.

When rabbits are injected intravenously with a quantity of virulent streptococci or pneumococci sufficient to cause death within two to four days the septicemia takes a definite course with slight variations. The bacteria rapidly decrease in number from the time of the injection to from two to four hours, at which time the blood is sterile or contains only a few bacteria. Within five to six hours the bacteria reappear in the blood and steadily increase until the death of the animal. If the bacteria are less virulent, the same quantity of culture causes a chronic type of infection. The same initial decrease in the number of bacteria occurs. The reëntrance into the blood is somewhat delayed, the septicemia does not reach the height obtained in the acute cases, and a second fall occurs within the course of a few hours. These rabbits show a low blood invasion or a sterile blood culture for several days. During this time they become emaciated to a marked degree. Then the low septicemia rapidly rises or the rabbit with a sterile culture develops a severe septicemia within a few hours and death takes place from a few hours to two days thereafter. In this type of infection local lesions, pericarditis, pleurisy, peritonitis, etc., are usually found. In the infections which run an acute course no gross lesions are found. If the bacteria are still less virulent they never reënter the blood after the initial disappearance and the rabbits remain in good condition. In order to obtain uniform results, the quantity of bacteria injected must not be so large that the bacterial substances

TABLE V.

Animal.	Time of bleeding and number of colonies.
Rabbit 1,	30 min. 3½ hrs. 22 hrs. 27 hrs. In 44 hrs. rabbit was found dead.
weight 1,450 gm.	150 0 × ×
Rabbit 2,	5 min. 30 min. 1½ hrs. 3 hrs. 4½ hrs. 7 hrs. 11 hrs. 21 hrs. 25 hrs. 28 hrs. 48 hrs.
weight 1,700 gm.	350 3 0 0 3 15 1 100 350 200 125
	72 hrs. 96 hrs. Dead 120 hrs. Autopsy showed pleurisy, pericarditis, and cellulitis over the ab-
	100 200 domen. Films from these places were heavily loaded with pneumococci.
Rabbit 3,	10 min. 4 hrs. 20 hrs. The blood remained sterile and the rabbit fully recovered.
weight 1,620 gm.	180 0 0

carried in are sufficient to cause an intoxication of the animal. If the quantity of bacteria injected is below this point the course of the infection depends largely upon the virulence of the infecting organisms. Yet variations in the natural resistance of individual animals may be sufficient to cause quite marked irregularities in the course of the infection. Pneumococci can be standardized so as to produce a particular type of infection more easily than streptococci. In general infections such as those produced by streptococci and pneumococci the number of the bacteria present in the circulating blood at a given time supplies accurate and delicate information regarding the severity of the disease. When the object is to determine the degree of virulence of bacteria, or of the efficiency of an experimental therapeutic method, the mere physical condition and mere death of the inoculated animals are not sufficient and satisfactory guides to the desired information. The death of the inoculated animal and the recovery of the infecting bacteria at autopsy do not give complete information concerning the intensity and course of the infection occurring during life. A large number of bacteria found in the blood and tissues at autopsy do not necessarily prove the existence of a heavy infection before the onset of the death agony, since it is a well known fact that bacteria multiply with enormous rapidity, once the natural resistance of the animal has been overcome. Therefore, if merely the life and death of the animal and autopsy findings must serve as our only guides, we shall lose much incidental information, perhaps of fundamental value. This may be especially true as regards the search for curative substances. Again, the individual animals of the same species, age, and apparently of identical physical condition react to the aggressive force of the infecting organisms variously. This fact is readily found out by the injection of a series of rabbits with lethal quantities of bacteria per body-weight, and by making tests at various periods before death results, which, in the case of streptococci, ranges from one to six days. Consequently a method which enables the determination of the degree and progress of the infection at any desired period is of obvious advantage.