FURTHER STUDIES ON VIRULENT PSEUDODIPHTHERIA BACILLI.*

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INTRODUCTION.

IN the *Journal of Infectious Diseases*, 1904, 1, p. 690, a variety of virulent pseudodiphtheria bacilli was described, 15 strains of which had been isolated from the throats of persons suffering from various diseases, including scarlet fever, measles and diphtheria. The 15 strains differed somewhat in minor characteristics, but all were virulent, and the pathological effect upon guinea-pigs was not neutralized by diphtheria antitoxin, but was neutralized by the serum of a rabbit immunized against one of the group. Most of the strains corresponded culturally and morphologically to the usual descriptions of pseudodiphtheria bacilli, but some resembled closely *B. diphtheriae*, from which they could be distinguished with certainty only through animal experiments. The term "virulent pseudodiphtheria bacilli" was applied to all these strains on the theory that they represented a distinct group within the large, loosely formed, class of organisms comprehended under the term "pseudodiphtheria bacilli."

It was found that the guinea-pigs injected with one of these organisms (the lethal dose being ½ to 1 per cent of the body weight) died usually within the following 24 hours, and that the changes found at autopsy differed from those found after the injection of *B. diphtheriae* in several respects; namely, absence of subcutaneous edema, absence of characteristic changes in the adrenals, more marked congestion of the liver and kidneys, presence of bacteria in large numbers in all organs and fluids. No soluble toxin was formed by the organisms, and the filtered cultures were entirely free from toxicity.†

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† After the publication of this work Dr. William Hallock Park called our attention to an article published in the *Proceedings of the New York Pathological Society* in 1898, by Louise Dudley Davis working in Dr. Park's laboratory, in which were described 30 strains of diphtheria-like organisms isolated from cases of scarlatinal otitis media, 12 of which proved pathogenic for guinea-pigs, producing in them general bacteriemia. Diphtheria antitoxin exerted no protection against these organisms. In all probability the bacilli described later by Dr. Ruediger (*Trans. Chicago Path. Soc.*, 1903, 6, p. 45) and many of those studied by us, are closely related to those isolated by Miss Davis.

The present article endeavors to take up the question as to the proper classification of these bacilli, and as to the nature of the protective serum.

CLASSIFICATION OF THE SO-CALLED VIRULENT PSEUDODIPHTHERIA BACILLI.

It may be taken for granted that any question which is discussed and settled each year for many successive years in different ways by different observers, is still an unsettled question, presenting unusual difficulties. Such is the question of the differentiation between the Klebs-Loeffler bacillus and the pseudodiphtheria bacillus. Hardly a year passes without bringing a number of articles on the subject of similarities and dissimilarities of the two organisms, and on the proper method of distingushing them. Over and over again a sure and satisfactory method is propounded, only to be rejected later as unsatisfactory in the hands of other observers. It is unnecessary to go into detail as to the varying views on this subject; for that, reference may be made to the article already mentioned. Sufficient to say that the scientific world is still divided into: first, the strict unitarians, who repudiate the term pseudodiphtheria bacillus altogether, and insist that there is but one organism; second, those that hold that the Klebs-Loeffler bacillus is one organism and the Hoffmann-Wellenhof bacillus quite distinct from it; third, those who look upon the latter as not a single bacillus, but a group of allied bacilli; fourth, those who insert a third group of "diphtheria-like" bacilli between *B. diphtheriae* and *B. pseudodiphtheriticus;* and, finally, those who hold that the term *B. diphtheriae* as well as *B. pseudodiphtheriticus* covers a group of organisms with several subdivisions.

In the face of this difference of opinion on the part of the most eminent authorities—each laboratory practically having its own special theory—it is no easy task to attempt to classify any doubtful strain of diphtheria-like bacilli according to the ordinary tests; i. e., on the basis of morphology, chemical activity, cultural characteristics, or virulence for guinea-pigs. By this we do not mean to assert that the typical pseudodiphtheria bacillus cannot be readily distinguished from the typical diphtheria bacillus. Such strains present, as a usual thing, little difficulty. It is the atypical strains which cause

the trouble—the short, solid forms of *B. diphtheriae*, growing abundantly and occasionally forming pigment;the diphtheria-like strains of pseudodiphtheria, growing scantily on agar, forming acid in broth, and resembling morphologically the Klebs-Loeffler bacillus. Occasionally, as in Nos. 5 and 11 of our strains, there is nothing in morphology or cultural characteristics which distinguishes these bacilli from typical diphtheria bacilli. In such cases, where the ordinary tests fail, a rational method lies at hand in the use of specific sera.

Spronck made use of such a method to show that the xerosis and pseudodiphtheria bacilli are absolutely distinct from *B. diphtheriae*, since the slightly virulent strains of the first two call forth the same symptoms in guinea-pigs protected by diphtheria antitoxin as in those not so protected, showing that their virulence is not due to the production of the specific toxin of diphtheria. Neisser and Glücksmann proved that animals treated with repeated injections of pseudodiphtheria bacilli are not thereby rendered immune to *B. diphtheriae*, and Petrie showed that filtered cultures of pseudodiphtheria bacilli are not capable of producing in horses immunity to the toxin of diphtheria. The objection to Spronck's method is that it can be applied only to organisms which possess some degree of virulence, and therefore not to the avirulent diphtheria bacilli, or to the great majority of pseudodiphtheria bacilli. If, however, it were possible to find a serum bactericidal to certain pseudodiphtheria bacilli, and not to *B. diphtheriae*, such a serum could be used as a means of differentiating the two classes of organisms in those cases in which morphological, chemical, and cultural tests have proved unsatisfactory. The bacilli which responded to the bactericidal serum would then be grouped in a large class, including varieties which might differ in virulence and, to a certain extent, in staining properties, acid production, etc.

In the article already referred to, details were given as to the animal experiments performed with 15 strains of virulent pseudodiphtheria bacilli. Guinea-pigs injected with broth cultures of these strains died from general bacteriemia, unless they were protected by the serum of a rabbit which had been immunized against one member of this group. The organisms were, therefore, shown to belong

to a class distinct from *B. diphtheriae*, for the protective serum was found to have no power to protect guinea-pigs againt injections of *B. diphtheriae*. Normal rabbit serum did not protect against a lethal dose of these bacilli.

Animal experiments of this kind are applicable only to virulent organisms, and in order to discover whether there were avirulent members of this same group of bacilli, it was necessary to substitute experiments in the test tube. The protective serum proved bactericidal *in vitro* as well as *in vivo*, and the test-tube experiments showed that normal rabbit serum also, although quite without protective action *in vivo*, had a transient but often marked bactericidal effect in the test tube. In the course of these experiments we tested the action of normal and immune rabbit serum on 71 strains of diphtheria and pseudodiphtheria bacilli. These included 13 of the original 15* strains; and in addition 10 strains of "diphtheria-like" bacilli kindly furnished by Dr. Anna Williams from the laboratory of the New York City Department of Health; seven strains of Wesbrook's Type D 2 sent by Dr. O. McDaniel from the laboratory of the Minnesota State Department of Health; and 41 strains of diphtheria and pseudodiphtheria bacilli which were isolated by various workers in this laboratory in the course of routine examinations of throat, nose, ear, and urine.

Out of all this material only five strains were added to the 13; that is, only five were shown to have the same receptors as the bacilli already described in the former article. Five more were found to be closely related to them, as they were killed by rabbit serum; but in the case of these last, the immune rabbit serum was no more strongly bactericidal than normal rabbit serum, while in the case of the five above mentioned the immune serum was much more strongly bactericidal than was normal serum. The other 48 strains were unaffected by the presence of rabbit serum, except when it actually appeared to favor their growth.

These 18 strains, then, form a distinct group, distinct from *B. diphtheriae* on the one hand, and from other pseudodiphtheria bacilli on the other. We have been unable thus far to find any single cul-

*No. 8 was overlooked and the cultures allowed to dry up. No. 10, which had apparently responded positively to the protective serum in the guinea-pig experiments, failed to respond in the test tube.

tural, morphological, or chemical characteristic which will distinguish some of them from the ordinary pseudodiphtheria bacilli, or others of them from diphtheria bacilli; the serum test seems the only positive one. We tested all our strains with reference to their behavior toward Gram's stain, their ability to hemolyze rabbit corpuscles, and their ability to ferment the dextrin serum-water medium of Hiss; but we can only say that our organisms resemble pseudodiphtheria bacilli rather more closely than diphtheria bacilli in these respects.

As to Gram's stain, it is notoriously unsatisfactory as applied to *B. diphtheriae*, for these bacilli are easily decolorized if left long enough in alcohol, and the method therefore gives varying results in the hands of different workers. Indeed, there are textbooks on bacteriology in which *B. diphtheriae* is placed among the Gram-negative organisms.[1] This want of agreement as to the staining properties of *B. diphtheriae* is usually explained on the grounds of faulty technique; those who obtained negative results are said to have used old cultures or to have kept their smears too long in alcohol.[2] Still there are not lacking reports of typical, virulent Klebs-Loeffler bacilli, which, in spite of most careful and approved staining methods, are decolorized by Gram (Zupnik,[3] Schick, and Ersetting[4]). It is admittedly a test which depends very largely on the personal equation, and is therefore of limited value. We have found few statements in the literature as to the behavior of pseudodiphtheria bacilli to Gram's stain, yet they are undoubtedly considered in general as Gram-positive bacilli, and we must admit that our results are unusual in this regard. Of the 18 strains which are killed by our immune serum, 11 are Gram-negative,* and seven Gram-positive. All of the five strains which respond equally to normal and immune rabbit serum are Gram-positive, and of the 48 strains which were unaffected by rabbit serum, all but four are Gram-positive. It may be argued that the Gram-negative bacilli should be relegated to another group, and that the term "pseudodiphtheria bacillus" cannot be applied to Gram-negative organisms. In reply to this, we can only say that we consider the characteristics which these strains

[1] PLAUT, *Deutsche med. Wchnschr.*, 1894, 20, p. 920.
[2] CZAPLEWSKI, *Hyg. Rundschau*, 1896, 6, p. 1029.
[3] *Prag. med. Wchschr.*, 1902, 271, p. 361. [4] *Wien. klin. Wchschr.*, 1903, 16, p. 993.
*Plaut's method was followed in staining our cultures (see reference above).

have in common with the Gram-positive pseudodiphtheria bacilli more important and significant than their failure to respond to a stain, which is generally looked upon as only a partially satisfactory test for these bacilli. The bacilli which are Gram-negative may be used to produce in rabbits a serum which both agglutinates and is bactericidal to the Gram-positive bacilli of this group.

It has been shown by several observers that the power to hemolyze rabbit's corpuscles is possessed by the great majority of strains of *B. diphtheriae*. According to Schwoner, 70 per cent is about the proportion of hemolyzing strains. The pseudodiphtheria bacillus is said by the same observer to be non-hemolyzing. All of our typical Klebs-Loeffler bacilli proved to be hemolyzers, while the typical pseudodiphtheria bacilli failed to hemolyze. As one would expect, some of the "diphtheria-like" bacilli (five in all) hemolyzed, but the majority (16) did not. This is, however, a test which must be used early, for many strains lose their hemolytic power after long growth on artificial media. Only two of the 18 organisms of our group hemolyzed rabbit corpuscles.

According to Knapp,[1] it should be possible to distinguish *B. diphtheriae*, *B. xerosis*, and *B. pseudodiphtheriticus* by fermentation tests, using the serum-water medium of Hiss with the addition of certain sugars. The first should ferment all but saccharose, the second all but dextrin, and the third none at all. We followed Knapp's procedure with much care, but were unable to confirm his results in any respect except as regards dextrin, and then only partially. The typical *B. diphtheriae* caused coagulation, or acid formation, or both, in the dextrin tubes in every case; the typical pseudodiphtheria bacillus did not. But in the case of the atypical forms (including Wesbrook's Type D 2), the results were various. We had five negative results as against 13 positive results, so that the test fails in the very cases in which it should be of use. In this case, too, the 18 bacilli of our group resemble pseudodiphtheria, for only two ferment dextrin. We have, thus far, found no organism which both ferments dextrin and hemolyzes rabbit's corpuscles, except typical *B. diphtheriae*.*

[1] *Jour. Med. Res.*, 1904, 12, p. 475.

* Since writing the above we have isolated from the throat of a case of diphtheria, along with a typical *B. diphtheriae*, an organism which grows like the less abundant forms of pseudodiphtheria bacillis is not virulent, is killed by our immune serum, and which ferments dextrin and hemolyzes rabbit', corpuscles.

The term "pseudodiphtheria bacillus" is loose and unsatisfactory. It may be that organisms represented by our 18 strains should be altogether separated from the pseudodiphtheria group and relegated to a class by themselves. Still, these bacilli correspond to the descriptions of pseudodiphtheria bacilli in the literature, having no distinctive feature except their virulence and their behavior to rabbit serum. All of this group which we have isolated, except two strains, were virulent for guinea-pigs immediately after isolation, and some of them are still so even after a lapse of over two years. Ordinarily the pseudodiphtheria bacillus is non-virulent, but virulent forms have often been reported. As to the effect of rabbit serum, it remains to be seen whether there is not a fair proportion of pseudodiphtheria bacilli which will be found sensitive to the action of normal rabbit serum. At the present stage of our work we are inclined to believe that the term "virulent pseudodiphtheria bacilli" is the most convenient and accurate designation for these organisms.

STUDY OF THE NATURE OF THE IMMUNE SERUM.

The bacteriolysin.—In immunizing our rabbits successive doses of broth cultures of *B. No. 1* (Ruediger) were injected under the skin at intervals of seven days, beginning with 1 c.c. of a 24 hour broth culture, and increasing by 1 c.c. each time until 7 to 12 doses had been given. The serum is decidedly bactericidal after the fourth dose, and may then be used to protect guinea-pigs, but the bactericidal substance increases if the doses are repeated up to about 10 or 12 doses. The individual rabbits vary a good deal in respect to immunization, some yielding a stronger serum after the seventh dose than others after the twelfth. There is nothing to be gained by increasing the amount given at each dose. After thorough immunization the serum of a rabbit retains its full bactericidal power for only about six weeks, after which it begins to lose its effect.

In making bactericidal tests the following method was used: For each strain five tubes were prepared, the first containing 1 c.c. of plain broth; the second, 0.8 c.c. of broth and 0.2 c.c. of normal serum; the third, 0.9 c.c. of broth and 0.1 c.c. of normal serum; and the fourth and fifth, the corresponding amounts of immune rabbit serum and broth. The tubes of each series were inoculated with the same quantity of bacterial culture, incubated, and plates were made at the end

of four to six hours, and again after 20 to 24 hours.* In some instances a normal rabbit serum would prove as strongly bactericidal as the immune serum if a large amount was used, say one part of serum to five of broth, and plates made only at the end of four hours; but if the two sera were diluted to 1:10 or 1:20, a decided difference always appeared in favor of the immune serum, and the 24 hour plates from all tubes usually showed that the action of the normal serum was transient.

Table 1 shows typical results in the use of the immune and normal sera.

The immune serum contains an agglutinin for these bacilli, yet the agglutination test is of little value in identifying the members of this group, owing to the fact that so many of them tend to clump in fluid suspension. Even a homogeneous suspension will often change after several hours in the incubator, and the tubes which contain no serum will appear as strongly agglutinated as those with serum. Especially is this true of strains which resemble closely *B. diphtheriae*, the so-called "diphtheria-like" bacilli. There were, however, several strains (Nos. 4, 5, and 9) which responded well to the agglutination test. Our immune rabbit serum agglutinated these in dilutions of 1:1,000 up to 1:5,000 according to the strength of the serum.

In the experiments given above, rabbit serum was used exclusively, but as it was desirable to obtain larger quantities of immune serum than rabbits could furnish, we immunized a goat also. The serum of goats is normally bactericidal, to these organisms more uniformly and in higher degree than is the serum of rabbits,† and the bactericidal substance increases very slowly with immunization. On the other hand, the agglutinins appear early and increase rapidly, so that the serum of an immune goat, which is no more highly bactericidal than it was before immunization began, is already strongly agglutinative, while the serum of an immune rabbit, which has increased more than tenfold in bactericidal power, is only moderately agglutinative.‡ We have had goat serum which agglutinated No. 9 in dilutions of 1:30,000, but no rabbit serum has ever agglu-

*As some of the strains agglutinate with the immune serum, it was thought that inaccurate results might come from using simply a loopful for plating, and we therefore tried plating the whole contents of the tube; but our results in these experiments did not differ from those obtained by the former method.

† Our work with normal goat serum is not yet complete, and we can only say that typical *B. diphtheriae* is not killed by normal goat serum. There are, however, pseudodiphtheria bacilli not belonging to our group which are killed by goat serum.

‡ It is evident that in the case of immune rabbit and goat serum it is not true that agglutinative power and protective power increase side by side and in relative degree as has often been asserted of other bactericidal sera.

TABLE 1.

EFFECT OF SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED WITH VIRULENT PSEUDODIPHTHERIA BACILLUS UPON VIRULENT PSEUDODIPHTHERIA, DIPHTHERIA, AND ORDINARY OR NONVIRULENT PSEUDODIPHTHERIA BACILLI.

BACILLI	NORMAL RABBIT SERUM				IMMUNE RABBIT SERUM						CONTROL	
	Serum 0.2 c.c. Broth 0.8 c.c.		Serum 0.1 c.c. Broth 0.9 c.c.		Serum 0.2 c.c. Broth 0.8 c.c.		Serum 0.1 c.c. Broth 0.9 c.c.		Serum 0.05 c.c. Broth 0.95 c.c.		Broth 1c.c.	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.
Virulent pseudodiphtheria bacillus (Ruediger No. 3, from throat of scarlet fever)	6,000	∞	6,000	∞	0	1	27	35	50	496	∞	∞
Virulent pseudodiphtheria bacillus (Ruediger No. 4, from throat of scarlet fever	4,224	∞	5,000	∞	24	1	34	6	26	0	4,000	∞
Virulent pseudodiphtheria bacillus (Hamilton No. 11, from throat in laryngitis)	400	7,000	200	7,100	25	700	8	600	30	330	560	6,000
Virulent pseudodiphtheria bacillus (Hamilton No. 16, from scarlatinal urine)	2,000	∞	2,510	∞	0	0	200	0	....	....	8,000	∞
Typical diphtheria bacillus (from diphtheric throat)	23	∞	21	∞	10	∞	37	∞	....	....	17	∞
" " " (" " ")	570	3,223	110	1,616	800	3,440	750	3,880	850	4,160	100	440
"Diphtheria-like" bacillus (A. Williams No. 9)	1,760	∞	....	....	1,840	∞	....	....	....	...	1,600	∞
Ordinary pseudodiphtheria bacilli No. 20 otitis media	560	....	600	840	720	440	2,000	1,000	....	....	4,500	10,000
" " " No. 27 conjunctivitis	600	∞	400	∞	350	∞	350	∞	480	∞	8,000	∞
" " " No. 31 scarlatinal throat	800	∞	8,000	∞	8,000	∞	8,000	∞	8,000	∞	750	∞
" " " No. 22 " "	1,440	∞	880	∞	1,280	∞	720	∞	....	....	2,000	∞

tinated in dilutions higher than 1:5,000. The immune goat serum, however, has so far not attained the bactericidal power of some rabbit sera. We have therefore used rabbit serum usually in testing the bactericidal action on cultures of diphtheria-like and pseudo-diphtheria bacilli, but in the study of the nature of the bactericidal substance we have used both goat and rabbit serum, and, in absence of a statement to the contrary, our conclusions apply to both kinds of serum.

All our experiments with immune serum, both of rabbits and of goats, have shown that the bactericidal substance is stable, resisting the deteriorating effects of age, light, heat, and even drying, to an unusual degree.

Deterioration from age takes place much more slowly when the blood is allowed to clot and the serum left in contact with the clot, than when it is removed by defibrinating and centrifuging the blood. To obtain a serum strongly bactericidal it is necessary to let it remain in contact with the clot for at least four hours. If it is to be kept for some time, it is better to remove it from the clot only as needed. By this method the serum, kept in contact with the clot in the ice-box, was found to be bactericidal after 32 days. This fact, that the presence of the clot postpones the deterioration of a bactericidal serum, has been noted by Ainley Walker, who also succeeded in reactivating old serum with the serum-free extract of fresh blood-clot. He concludes that "complement is formed by leucocytes and becomes liberated into plasma or serum by their disintegration," and thus the disintegrating leucocytes supply continually fresh complement and prevent deterioration of the serum. Whether or not Walker's explanation of the phenomenon is correct, we have found his statement to be true in the case of our immune serum.

One week's exposure to strong daylight, not necessarily sunlight, weakens, but does not entirely destroy, the bactericidal power of these sera.

Zero temperature partially inhibits the action of the bactericidal sera, but not entirely. The usual mixture of serum and culture was placed in a tube, and the tube thrust into a pan of powdered ice. A loopful plated at once gave 8,000 colonies, while the same dilution at 37° C. gave only 388. The same serum, however, became active again

when placed in the incubator, and a plate from the same tube, at the end of 24 hours at 37° C., had only 720 colonies. If the bacilli are removed from the serum, washed, and then incubated, they will be found to have absorbed the bacteriolysin while at 0° C.—a point which will be referred to later on.

Filtration through a porcelain filter removes the active principle concerned in bacteriolysis and the filtrate is inactive, provided a very fine filter is used, otherwise a part of the bactericidal substance will pass through. The bacteriolysin of normal goat serum can be removed in the same way.

The serum can be evaporated to dryness without losing its bacteriolytic power.

When we proceeded to test the effect of temperature, we found that the resistance to heat, like the resistance to age, is much greater than that usually found in bactericidal serum. Room temperature is well borne, provided the serum is protected from light, and a week's exposure to 37° C. is not sufficient to produce complete inactivation. Exposure to 58° C. for 30 minutes has no effect upon the bactericidal power, and to 65° C. for 30 minutes produces only a slight loss. When, however, the time of exposure is increased to two hours, a gradual loss of power occurs beginning at 60° C. and continuing to the point at which the serum, diluted 1 : 4 with normal salt solution, coagulates, about 85° C.

Somewhere between 80° and 90° C. the bactericidal substance is destroyed. Two hours' exposure to 80° C. is not always sufficient to destroy it in perfectly fresh, strongly bactericidal serum, but an hour's exposure to 85° C. coagulates the diluted serum, and the fluid expressed from the coagulum is inactive. Serum heated to 70° C. for two hours is no longer capable of protecting guinea-pigs against a lethal dose of pseudodiphtheria bacilli.

All these experiments, except the last mentioned, were made with both goat and rabbit serum, and the two gave the same results. The normal serum of both these animals differs from the immune in resistance to heat, losing its bactericidal power by exposure to 70° C. for two hours. The difference in heat resistance of normal and immune serum has been noted before. Ehrlich and Morgenroth[1]

[1] *Gesammelte Arbeiten zur Immunitätsforschung*, Berlin, 1904, p. 21.

found, for instance, that the hemolysin for sheep's corpuscles present in normal goat serum was destroyed at 57° C., but the hemolysin in the serum of a goat immunized with sheep's blood could resist 65° C. with only a slight diminution of its power. This heat-resisting complement was by them supposed to be present in normal goat serum, in small amounts, and to be increased by immunization.

The possibility must always be considered that a bactericidal serum, which is very resistant to high temperature, owes its power to a strong alkaline reaction. Thus Hamburger has increased the alkalinity of blood by passing CO_2 through it, and found it to be more strongly bactericidal in consequence. Emmerich claims that he has reactivated inactivated dog serum with sodium hydroxide, and Fodor[1] believes that the increased alkalinity of rabbit serum after immunization against anthrax is responsible for its bactericidal power. However, in the case of our immune serum, increased alkalinity can be ruled out as a factor in the increased bactericidal power, for titration shows that there is no difference between the reaction of normal and that of immune rabbit serum.

To sum up then : The immune serum is not more alkaline than is normal serum. It is very resistant to heat, to room or incubator temperature, to age, to cold, and to drying. It is partly or wholly inactivated by filtration. The question as to the exact nature of the bacteriolytic substance is not easy to answer, but we incline strongly to the view that the active principle is a single substance rather than complement and amboceptor. We have never been able to separate the active principle into two bodies by any method thus far attempted. The high degree of heat resistance and of resistance to age certainly argues against the presence of complement, especially when it is found impossible to reactivate the heated serum or the old serum. Our experiments in reactivation have been unsatisfactory, owing somewhat, perhaps, to the fact that both heated serum and normal unheated serum are bactericidal to a slight degree, and therefore it is difficult to estimate results. We have tried repeatedly to restore the complement to heated serum, but have never yet obtained indubitable results. It might be argued that the intermediary body also is destroyed at 80° C., and that this is the explanation of the failure of

[1] References given in KOLLE U. WASSERMANN, *Hb. d. path. Mikro-Organ.*, 1904, Bd. IV, p. 560.

reactivation experiments, but that would be to assume equal stability in complement and amboceptor; indeed, it would necessitate an entirely new conception of complement.

The attempt to remove the amboceptor by absorption resulted in the absorption of the bactericidal substance *in toto*, just as the effort to inactivate by heat resulted in permanent inactivation. Bacilli were treated with immune serum at zero, were then centrifuged, washed three times, and placed in the incubator, some with normal salt solution only, others with different quantities of normal rabbit serum. There was no difference in the plates made from the tubes, except what could be accounted for by the slight bactericidal action of the normal serum.

In a recent study of the anthracidal substance in the serum of the white rat made by one of us,[1] the properties of the anthracidal substance in the serum of adult rats were found to correspond in several ways with those of our bactericidal serum. It was found that, while the anthracidal substance in the serum of young rats is thermolabile, the same cannot be said of the substance in the serum of older rats. In the latter the anthracidal substance is very stable, resisting exposure to a temperature of 66° C., resisting direct daylight for nine days, and the temperature of the incubator for three weeks, before complete destruction, and the temperature of the ice-box for the same period without any loss of power at all. The activity is partially suspended at 0° C. Alkalinity seems to have nothing to do with bacteriolysis, for the heated and unheated sera have the same degree of alkalinity. The conclusion drawn as a result of these observations was the same as that we have sustained in regard to our goat serum and immune rabbit serum; namely, that the anthracidal substance in the serum of adult rats is simple and indivisible.

Our experiments with the immune serum in regard to its effect upon phagocytosis confirm the conclusions reached in the experiments in bacteriolysis outlined above, as they show that we have here a specific serum which acts in a certain way toward the organisms belonging to the same group as the one used to produce immunity, and not toward other pseudodiphtheria bacilli or toward the diphtheria bacillus. The experiments also indicate that phagocytosis

[1] HORTON, *Jour. Infect. Dis.*, 1906, 3, p. 110.

plays an important rôle in the protection of guinea-pigs against these pseudodiphtheria bacilli.

In the course of our experiments on the action of immune serum in guinea-pigs injected with a lethal dose of *B. No. 4*, we had occasion to examine the blood of these animals and compare it with that of normal guinea-pigs, and with that of guinea-pigs injected with the same amount of culture, but without immune serum. When two guinea-pigs are given each a fatal dose of *B. No. 4*, and guinea-pig B is given a dose of protective serum, while A is not, the blood of A will show an increase of polymorphonuclear leucocytes as compared with the blood of a control animal, but the blood of B will show a far greater increase as made clear in the following differential count.

DIFFERENTIAL BLOOD COUNT.

	Per Cent Polynuclear	Per Cent Small Mononuclear	Per Cent Large Mononuclear
Control Guinea-pig	1.6	82.0	16.0
Guinea-pig A–2.5 c.c. broth culture *B. No. 4*	8.0	87.0	4.0
Guinea-pig B–2.5 c.c. broth culture *B. No. 4* + 2c.c. immune serum	44.0	44.0	11.0

Such an increase in the number of polymorphonuclear leucocytes as a result of the injection of the immune serum points certainly to an increased leucocytic activity under the stimulus of the serum.

That phagocytosis is dependent upon a substance in the serum has been shown by Wright and Douglas. In the study of the effect of our immune serum on phagocytosis the technique followed was the same as that followed by Hektoen and Ruediger.[1]

The blood was drawn by means of a sterile glass syringe directly from the heart of the lower animals, and from the vein at the bend of the elbow in human beings. The washed corpuscles were added to tubes containing serum and a suspension of bacilli in normal salt solution, in the proportion of 0.25 c.c. of corpuscles to 0.25 c.c. of serum and 0.5 c.c. of bacterial suspension. We used the corpuscles of the dog, goat, guinea-pig, and rabbit — in the last case obtaining polymorphonuclear leucocytes by injecting aleuronat suspension into the pleural cavity and withdrawing the exudate — and also of 12 healthy adults. In each instance control tubes were prepared, one containing no serum at all, only normal salt solution; the second containing normal rabbit serum, and to these was often added a third tube containing the serum homologous to the corpuscles. After incubation for one hour at 37° C., smears were made from the tubes, stained with Leishmann's stain, and examined for phagocytes.

[1] *Jour. Inject. Dis.*, 1905, 2, p. 128.

Nine experiments were made with leucocytes of lower animals, and 40 with human leucocytes. Without exception, in all of these experiments the presence of our immune serum favored phagocytosis of this group of bacilli, but had no effect on phagocytosis of *B. diphtheriae*, or of six strains of ordinary pseudodiphtheria bacilli. There was practically no phagocytosis in the tubes containing no serum, there was a varying amount in the tubes with homologous serum, but in the experiments with the virulent pseudodiphtheria bacilli there was always a marked increase in the amount of phagocytosis in the tubes with immune serum as compared with normal rabbit serum. Table 2 shows this, the results being stated in figures which indicate the average number of bacilli found in a count of 40 polymorphonuclear leucocytes.

TABLE 2.

PHAGOCYTOSIS OF VIRULENT PSEUDODIPHTHERIA BACILLUS UNDER THE INFLUENCE OF THE SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED AGAINST VIRULENT PSEUDODIPHTHERIA BACILLI.

Washed Corpuscles in NaCl Sol.....0.25 c.c. Serum or NaCl Sol.....0.25 c.c. Bacterial Suspension.....0.50 c.c.	PHAGOCYTOSIS (AVERAGE IN 40 LEUCOCYTES)			
	NaCl Sol.	Homologous Serum	Normal Rabbit Serum	Immune Rabbit Serum
Guinea-pig leucocytes (blood).....	0.1	1.5	1.5	5.5
Goat " "	0.1	1.9	1.5	9.5
Rabbit " (exudate).....	1.8	...	6.0	7.8
Dog " (blood)	1.0	3.5	13.5	22.0
Human " "	2.8	11.4	4.3	20.7

Immune goat serum also causes marked phagocytosis of virulent pseudodiphtheria bacilli by human leucocytes, while normal goat serum had but little opsonic effect, the average in three different experiments not exceeding 1.7 per leucocyte.

In most of the experiments *B. No. 1*, which had been used for immunization, was used also for testing phagocytic power, but it was observed that the same results were obtained with *B. No. 4* (Ruediger) and *Nos. 5, 9, 11*, and *16* (Hamilton). It must be added that the sera of five immune rabbits and of more than a dozen non-immune rabbits were used in these experiments, so that an individual peculiarity in the serum of any one animal can be ruled out.

An identical series of tubes was prepared at the same time, and a suspension of *B. diphtheriae* was substituted for the pseudodiph-

theria bacillus, in order to ascertain whether the opsonin present was specific for the latter. The result is illustrated in Table 3:

TABLE 3.

PHAGOCYTOSIS OF DIPHTHERIA BACILLI UNDER THE INFLUENCE OF THE SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED AGAINST VIRULENT PSEUDODIPHTHERIA BACILLI.

Washed Corpuscles in NaCl Sol. .....0.25 c.c. Serum or NaCl Sol. .....0.25 c.c. Bacterial Suspension. .....0.50 c.c.	PHAGOCYTOSIS (AVERAGE IN 40 LEUCOCYTES)			
	NaCl Sol.	Homologous Serum	Normal Rabbit Serum	Immune Rabbit Serum
Guinea-pig leucocytes (blood)	0.0	1.8	0.6	0.87
Goat " "	0.0	4.4	2.3	3.0
Dog* " "	9.9	40.0	40.0	40.0
Human " "	1.4	7.8	4.0	2.7
Human* " "	0.5	40.0	40.0	40.0

* Leucocytes so filled with bacilli that no accurate count could be made.

As shown in Table 3, there is no difference in the opsonin of immune serum and that of normal serum as regards diphtheria bacilli.* Similar results were obtained with six strains of typical pseudodiphtheria bacilli (Table 4). The opsonin is therefore specific for the virulent group of pseudodiphtheria bacilli, just as the bactericidal substance is specific for them. It is well in this connection to emphasize the fact that the same effect is produced in both animals, the goat and the rabbit, by immunization. In the serum of both there appears a specific opsonin and a specific bacteriolysin which have the same degree of thermostability and of resistance to age and light.

TABLE 4.

PHAGOCYTOSIS OF TYPICAL, NON-VIRULENT, PSEUDODIPHTHERIA BACILLI UNDER THE INFLUENCE OF THE SERUM OF NORMAL RABBITS AND OF RABBITS IMMUNIZED AGAINST VIRULENT PSEUDODIPHTHERIA BACILLI.

Washed Human Corpuscles in NaCl Sol. .0.25 c.c. Serum or NaCl Sol. .....0.25 c.c. Bacterial Suspension. .....0.50 c.c.	PHAGOCYTOSIS (AVERAGE IN 40 LEUCOCYTES)		
	NaCl Sol.	Normal Serum	Immune Serum
No. 20, diphtheria—ear	0.1	5.09	4.4
No. 27, conjunctivitis—eye	0.2	1.5	0.85
No. 34, measles—ear	0.7	4.2	4.7
No. 31, scarlet fever—throat	0.0	0.45	0.9
No. 6, measles—throat	0.7	11.0	4.0

*These results obtained with *B. diphtheriae* are not in accordance with those of Wright and Douglas who state that *B. diphtheriae* is not taken up by human leucocytes, but they are in accordance with the results recently published by Walkerr (*Jour. Med. Res.*, 1905, 13, p. 173). In his experiments—made chiefly with the leucocytes of one individual—there was always phagocytosis of *B. diphtheriae*, although never to the extent shown in two of our cases. The individual variation is apparently very great in the case of *B. diphtheriae*

In course of these experiments several points of difference have appeared between the bactericidal substance in our sera and the opsonin, showing that they are not identical. In the first place opsonin disappears almost completely after heating immune rabbit serum at 70° for two hours and from immune goat serum after heating at 70° degrees for one hour, while the bacteriolysin persists in immune serum heated at 80°. This persistence of the bacteriolysin makes it impossible to prove destruction of bacilli by phagocytosis *in vitro* as might be done were the bacteriolysin destroyed at a lower temperature than the opsonin instead of a higher. In the second place, a serum strongly bactericidal may have little opsonin. Normal goat serum is rich in bacteriolysin, but has almost no opsonin, while immune goat serum has acquired a large amount of opsonin without any increase in bacteriolysin. Normal human serum, which is only slightly bactericidal, may contain a large amount of opsonin.* So far as our experiments go, it appears that the opsonin for these bacilli is not composed of a complement-amboceptor complex.

Just as the bacteriolysin in normal goat serum was found to be less resistant to heat than that in immune serum, so the opsonin in normal serum is less resistant than that in immune serum. For a perfectly satisfactory proof of this one would have to compare two specimens of rabbit or goat serum, normal and immune, but these normal sera contain so very little opsonin that no striking results can be obtained with them. The opsonin in human serum is destroyed at a far lower temperature than that in the serum of immune goats and rabbits; namely, at 56° to 58° C. for 30 minutes.†

TABLE 5.

THE EFFECT OF HEAT UPON THE OPSONIN FOR VIRULENT PSEUDODIPHTHERIA BACILLI.

Mixtures		Phagocytosis (40 Leucocytes Counted)
Bacterial suspension 0.5 c.c. +washed human corpuscles 0.25 c.c.+	Immune rabbit serum 0.25 c.c.	16.3
	Heated at 70° C. 2 hrs. 0.25 c.c.	1.8
	Immune goat serum 0.25 c.c.	11.7
	Heated at 70° C. 1 hr. 0.25 c.c.	1.0
	Normal human serum 0.25 c.c.	19.3
	Heated at 56° C. 30 min. 0.25 c.c.	1.65

* We have not tested many specimens of human serum for bacteriolysin, and are unable to say whether or not there is a decided individual variation in this respect.

† We have not tested the heat resistance of the opsonin for virulent pseudodiphtheria bacilli by Dean's method, *Proc. Roy. Soc.*, 1905, Series B, 76, p. 506.

NATURE OF THE PROTECTIVE ACTION OF IMMUNE SERUM.

In view of the unvarying results obtained in the test-tube experiments in phagocytosis, it would seem probable that the same process occurs in the animal body and that the immune serum protects guinea-pigs against the virulent pseudodiphtheria bacilli by increasing phagocytosis as well as by direct bacteriolysis. We have said that guinea-pigs, dying after injection with a fatal dose of virulent pseudodiphtheria bacilli, yield growths of the organism from peritoneal and pleural fluids, from urine, heart's blood, lungs, liver, spleen, and kidneys. Three hours after inoculation this general invasion has taken place, for cultures made at this time all yield growths. This is true of animals protected by immune serum as well as of those not so protected. If both animals are killed at the end of three hours, a general infection will be found to have occurred in both, but the number of bacilli in smears from the peritoneal exudate of the one which received protective serum is not nearly so great as in the case of the unprotected guinea-pig, and the bacilli are agglutinated. After three hours a progressive diminution of bacteria occurs in the body of the protected guinea-pig. In the animals killed at the end of six hours, cultures are obtained from the spleen and peritoneal cavity only; and in those killed at the end of 18 hours all cultures remain sterile. The bacilli evidently multiply rapidly and invade the general circulation within three hours after inoculation, even in the presence of protective serum. Destruction of bacilli has begun, however, in the presence of immune serum, during the first three hours, and from then on proceeds rapidly, being well advanced at the end of six, and complete at the end of 18, hours. The results of experiments in the test tube appear to correspond with those in in the animal body.

TABLE 6.

PROGRESSIVE BACTERIOLYSIS *in vitro*.

Suspension of *B. No. 6*	0.75
Immune serum	0.25
One loopful plated at once	33
" " " after 1½ hrs.	1,200
" " " " 3 "	800
" " " " 4½ "	560
" " " " 6 "	52
" " " " 18 "	36

We have already spoken of the increase of polymorphonuclear leucocytes in the blood of protected guinea-pigs as compared with those not so protected. There is also a much greater number of leucocytes in the peritoneal fluid of protected animals, as shown in the following experiment:

Guinea-pig A was killed three hours after receiving intraperitoneally 4 c.c. of a broth culture of *B. No. 4*. The exudate was almost clear with many bacilli and but few leucocytes, 2 per cent of which were polynuclears, 94 per cent small mononuclears, and 4 per cent large mononuclears.

Guinea-pig B received, in addition to 4 c.c. of broth culture of *B. No. 4*, 2 c.c. of immune serum. It was killed at the end of three hours. The peritoneal exudate was slightly cloudy, and contained few bacilli and many leucocytes, 54 per cent of which were polynuclears, 28 per cent small, and 12 per cent large mononuclears.

So large an increase of polymorphonuclears in the blood and in the exudate certainly would seem to point to phagocytosis, but we must acknowledge that we were never able to demonstrate any considerable number of phagocytes in smears from the blood or from the organs of our protected guinea-pigs; in the peritoneal exudate, however, quite marked phagocytosis takes place.

An animal which was given immune serum and a lethal dose of *B. No. 4*, was killed at the end of six hours. Cultures showed that bacilli were present only in the spleen and peritoneal fluid. Smears from the peritoneal fluid showed no active phagocytes at all; smears from the spleen showed that of 75 polymorphonuclear leucocytes only 13 were engaged in phagocytosis, and these contained an average of four bacilli to the phagocyte.

Another guinea-pig was given the usual dose of broth culture and immune serum, and a small amount of fluid was withdrawn from the peritoneal cavity by means of a syringe every 30 to 40 minutes for two and a half hours after injection.

The following results were obtained:

After ½ hour	no polymorphonuclear leucocytes
" 1 "	average of 20 leucocytes= 8.7 bacilli
" 1 hr. 40 min.	" " " = 6.7 "
" 2 hrs. 25 "	" " " =13.4 "

A second guinea-pig treated in the same way at the same time was killed at the end of three hours. The peritoneal fluid contained 54 per cent of polymorphonuclear leucocytes, but they averaged only 1.9 bacilli to the polymorphonuclear leucocyte. There were no phagocytes in the blood or in the smears from any organ except the spleen, where a few were seen.

Phagocytosis in the peritoneal cavity is apparently at its height about two hours or two hours and a half after injection, and the average of bacilli found so far in the leucocytes at that time is 13 to the polymorphonuclear leucocyte. Now the almost complete

absence of phagocytosis in the organs and in the blood, which contained bacilli as shown by cultures, might appear to throw some doubt upon the importance of the part played by phagocytes in the action of our immune serum, at least so far as ridding the blood of bacilli is concerned. It may be, however, that we have not yet found the right moment for making search for phagocytes. It should be noted that so far we have neglected to study carefully the bone marrow from this point of view.

SUMMARY.

1. The bacilli described by us under the name of "virulent pseudodiphtheria bacilli" form a group distinct from *B. diphtheriae* on the one hand and from other pseudodiphtheria bacilli on the other.

2. The distinction lies in the fact that goats and rabbits, immunized against one member of this group, yield serum which is bactericidal to the others of the group, but not to *B. diphtheriae* or to other pseudodiphtheria bacilli, and which contains opsonin specific for the members of this group.

3. The study of the bacteriolysin and opsonin of our immune serum reveals facts which should be of general interest to immunologists, inasmuch as we have here an immune serum of high degree of thermostability, apparently not containing complement and amboceptor.

4. Experiments on guinea-pigs indicate that the immune serum causes a marked polynucleosis, and increases phagocytosis *in vivo* as well as *in vitro;* hence the protective action of this serum must be ascribed in part at least to the immune opsonin.

6. The bacteriolysin and opsonin of these immune sera are not the same substance.

In the future we hope to revert to the question as to the frequency of "virulent pseudodiphtheria bacilli," and to take up again their clinical significance.