TOXIN AND ANTITOXIN OF AND PROTECTIVE INOCULATION AGAINST BACILLUS WELCHII.

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This study of the pathogenicity of infection by the group of *Bacillus welchii* has followed from several fortuitous circumstances. First, there was the insistent problem, only partially solved by the improvement in the antiseptic treatment of wounds, of gas bacillus infection following shell and bullet wounds everywhere in the war; second, there were available to us several cultures of *Bacillus welchii* isolated during the summer of 1916 on the western battle front by Dr. Simonds, who kindly presented them to the Institute; and finally and especially, Dr. Flexner's wish that with these cultures the subject of gas bacillus infection of the pigeon which he had observed many years before at the Johns Hopkins Hospital should be reinvestigated, as, in his opinion, the process in that species of animal epitomized the pathologic effects occurring in gas gangrene in man, and because he believed that a better understanding of the one condition would serve to explain many still obscure points in the other.

It will be of interest in this connection to review briefly certain facts concerning gas bacillus infection in the pigeon, since the condition is one little known to pathologists and bacteriologists. The classical article by Welch and Nuttall[1] on the gas bacillus appeared in 1892. It was followed by a paper on gas bacillus infection in man by Welch and Flexner[2] in 1896. The latter article was incomplete,[3] and the concluding part which was to appear in the next number was never published. The second paper was to deal more particularly with experimental gas bacillus infection in animals—in the guinea

[1] Welch, W. H., and Nuttall, G. H. F., *Bull. Johns Hopkins Hosp.*, 1892, iii, 81.

[2] Welch, W. H., and Flexner, S., *J. Exp. Med.*, 1896, i, 24.

[3] Personal communication from Dr. Flexner.

pig and pigeon particularly. Since the pigeon had proved to be highly subject to infection and to respond with characteristic pathologic reactions, that animal came to be employed by the laboratory staff in the more or less routine study of the gas bacillus. But because of the circumstances stated, no full and sufficient description of the local lesions in the pigeon, which in disorganizing effect are comparable with the destructive lesions sometimes present in man, came to be published until some years later, when, at Dr. Flexner's suggestion, Dr. Herter,[4] then engaged in the study of the *Bacillus welchii* group of bacteria occurring in the alimentary tract, employed this animal for inoculation. The lesions as described by Herter agree closely with those present in our pigeons inoculated with cultures of the bacilli.

Sources and Nature of Cultures.

The main part of our experiments has been made with five strains of *Bacillus welchii*, of which four were obtained through the kindness of Dr. Simonds. The fifth was isolated by us from a piece of clothing which had long been worn. The history of the Simonds cultures follows. The tests given were made by him.

Strain 365 a.—Isolated, Aug. 13, 1916, from scrapings from a bullet wound of the thigh, which showed a moderately severe gaseous gangrene. Its virulence for laboratory animals had not been tested.

Strain 386 cd.—Isolated, Sept. 9, 1916, from a fragment of shell with adherent bits of clothing, removed from a wound of the thigh. The patient did not develop gaseous gangrene. The organism had not been pathogenic for guinea pigs.

Strain 617 d.—Isolated, Aug. 27, 1916, from a case of violent gaseous gangrene following a bullet wound of the thigh with injury to and subsequent ligature of the large vessels of the leg. The limb was amputated. Although the stump was gaseous, the patient recovered. This strain is very pathogenic for guinea pigs, producing typical lesions and killing the animal in less than 24 hours after subcutaneous injection of 0.5 cc. of a 24 hour dextrose broth culture.

Strain 669 b.—Isolated, Aug. 21, 1916, from a case of gaseous gangrene following a bullet wound which caused shattering of the lower end of the femur. The leg was amputated; the patient recovered. Injection of 0.5 cc. of a 24 hour dextrose broth culture subcutaneously into a guinea pig was followed by a local-

[4] Herter, C. A., Bacterial infections of the digestive tract, New York, 1907, 198.

ized gaseous gangrene and sloughing of the skin and subcutaneous tissues. The animal died 2 weeks after the injection. No gas bacilli were found at autopsy in films from tissues adjacent to the slough.

The history of our own strain is as follows: A piece of cloth from the lining of an old overcoat was thrust with tissue forceps deep into the right breast muscle of an anesthetized pigeon. The next morning the wounded muscle was greatly swollen, and crepitation was present. Pressure near the wound forced out gas bubbles which ignited with a snap. The pigeon was drooping and died at 2.30 p.m.

Autopsy.—The skin over the right side was edematous and covered with blebs. There was a reddish brown gelatinous exudate in the subcutaneous tissues of both groins and extending over the right pectoral muscles. A film preparation made from this exudate contained a few plump Gram-positive bacilli. The inoculated muscle was edematous and necrotic, most pronounced about the cloth, but extending along the muscle sheaths to the insertion of the fibers. Films from the necrotic muscle contained many plump Gram-positive bacilli and a few Gram-positive diplococci and Gram-negative bacilli.

Tubes of recently boiled litmus milk, blood agar, and blood bouillon were inoculated with the subcutaneous exudate and necrotic muscle. After 24 hours' incubation, the milk tubes showed the so called "stormy fermentation," and those inoculated with the subcutaneous exudate contained a plump Gram-positive bacillus in pure culture. The tubes from the necrotic muscle contained a similar bacillus, together with Gram-positive cocci in pairs and short chains and a Gram-negative bacillus. The blood media inoculated with the subcutaneous exudate remained sterile, while those of the necrotic muscle yielded cocci and Gram-negative bacilli only, the latter proving to be a variety of *Bacillus coli*. We shall call the culture of the Gram-positive bacillus, to be described more fully later, P-50.

The five cultures of Gram-positive bacilli enumerated have been tested for motility, spore formation, quantitative acid and gas production, liquefying action on gelatin at 22° and 37°C., pathogenicity for guinea pigs, rabbits, and pigeons, and still other properties. For example, the several strains have been tested for agglutination and lysis in normal rabbit and guinea pig sera, for hemolysins, for gas production in rabbits after the method of Welch and Nuttall, and for agglutination in artificially produced immune sera. As far as these tests are indicative, they place all five cultures among the group of *Bacillus welchii;* so far as they relate to specific properties, *e.g.*,

specific and cross agglutination, they indicate certain differences among them such as have commonly been observed among members of the group.

Pathologic Effect in Animals.

The feature of most importance among the properties just given which define this group of bacilli is pathogenic effect. This is true in the first place because of the similarity of certain of the lesions in animals to those arising in gaseous gangrene in man; and next and especially, because the investigation of the manner in which the lesions arise in animals led us to the discovery of the conditions under which a highly potent soluble toxic agent is regularly produced by the bacilli on which their poisonous or lethal action chiefly if not wholly depends.

In the past, the laboratory animal usually employed to test pathogenicity of *Bacillus welchii* has been the guinea pig. Many more cultures produce mild local infections, from which recovery takes place, than severer ones which are fatal. But the subcutaneous or intramuscular injection of cultures gives rise to local swelling, gas production, liquefactive necrosis of the involved muscle and skin, and, if the animal survives, eventual sloughing and cicatrization.

The rabbit is more resistant to local infection than is the guinea pig, but nevertheless lesions can be produced by active cultures which in general resemble those of the guinea pig. The rabbit reacts either to more highly virulent cultures or to larger doses of less effective ones. In the case of the former, death may result. But in both the rabbit and the guinea pig general blood invasion, even when the course of the infection is lethal, either does not take place at all or so few bacilli enter the blood that the cause of death cannot be attributed to a septicemia.

The peculiar susceptibility of the pigeon to infection with *Bacillus welchii* has not been utilized to the degree deserved. For in no other laboratory animal is infection produced so readily and with such a wide number of cultures, and in no other does the pathologic process proceed so swiftly and characteristically and with effects so nearly resembling the condition of gaseous gangrene in man. Probably this lack of use of the pigeon is to be ascribed to the rather brief refer-

ences made to the subject in the literature, which is almost confined to a few summary statements emanating from the Johns Hopkins Hospital at about the period already referred to. Dr. Flexner himself employed mainly blood containing large numbers of the bacilli for inoculating pigeons. In some instances the blood was taken post mortem from human cases, and in others from rabbits injected, killed, and incubated by the Welch-Nuttall method. Inoculation from pigeon to pigeon was also carried out. The animals succumbed at periods ranging from 5 to 24 hours. The autopsies revealed lesions precisely like those already described (page 121), except that the injected blood would often remain to tinge the tissues. The bacilli were very numerous in the disorganized muscle, fewer in the gelatinous exudate, and very few in or absent from the heart's blood.

The five cultures were pathogenic for pigeons, although not equally so. Simonds' Culture 617 d proved most virulent, not only for pigeons, but for rabbits and guinea pigs also. Doses of 0.01 to 0.05 cc. of a 20 hour glucose broth culture injected directly into the breast muscles were invariably fatal to pigeons in 6 to 20 hours. 0.0005 cc. of such a fluid culture put upon a bit of gauze and introduced into the muscles usually but not invariably produced fatal infection. Of Cultures 365 a and 669 b approximately 0.1 cc. of a glucose broth culture was the minimal lethal dose; while the dose of Cultures 386 cd and P-50 required to produce corresponding effects was 0.2 to 0.3 cc. The minimal lethal doses for guinea pigs and rabbits were several times those used for the pigeons.

Cause of Death in Bacillus welchii Infection.

In man, infection with *Bacillus welchii* tends to be a local process, even when severe, and invasion of the general blood occurs if at all only during the death agony or post mortem. In a small number of instances in man general infection seems to have played an important part in causing or hastening death.[5] But as these cases are the exception, even when death occurs, in man as well as in the pigeon, rabbit, and guinea pig, it may be assumed that soluble chemical substances entering the circulation from the local lesion bring about

[5] Thaon, P., *Compt. rend. Soc. biol.*, 1908, lxiv, 863.

the severe symptoms and the fatal termination. The question at once arising from this general consideration relates to the probable nature of the poisonous bodies. Several possibilities present themselves: They may arise from the bacilli, they may be yielded by the disorganizing tissues, or they may be of the nature of acids which disturb profoundly the hydrogen ion concentration of the body fluids. The protocols which follow are given to show the manner in which death may be produced in rabbits and pigeons under circumstances in which the blood remains wholly or practically free of the bacilli.

Experiment 1.—Pigeon. 11 a.m. 0.2 cc. of a glucose broth culture of Culture 617 d was injected into the breast muscle. 6.30 p.m. Animal died. Immediate autopsy. Usual gaseous, gelatinous, and necrotic local lesions containing myriads of bacilli. Films from the heart's blood and peritoneal fluid showed no bacilli, although a few were present, as a glucose broth culture was positive.

Experiment 2.—Pigeon. 11 a.m. 2 cc. of broth culture of Culture 617 d injected into breast muscles. 2.30 p.m. Animal died, having survived $3\frac{1}{2}$ hours. Local lesions similar to the preceding experiment, but the films from heart's blood contained a few bacilli.

These experiments were varied and repeated with different doses of the culture, but in no instance did a severe septicemia develop.

Experiment 3.—Rabbit. 11 a.m. Sedimented bacilli from 10 cc. of a 24 hour glucose broth culture of Culture 365 a were injected into the ear vein. 12 m. Films from the heart's blood showed a few bacilli, and the culture was positive. 9.30 p.m. Animal stuporous and breathing heavily. 10 p.m. Films and cultures from heart's blood negative. 10.20 p.m. Died. Immediate autopsy showed a dark and large spleen, but blood intact and serum clear. Films and cultures yielded no bacilli from the blood but some from the spleen and liver.

Experiment 4.—Rabbit. Mar. 14, 1917. 11 a.m. Sedimented bacilli from 2 cc. of broth culture No. 617 d given intravenously. Mar. 19, 8 a.m. Dead. Autopsy showed bloody fluid in peritoneum and small amount of clear fluid in pleura. The former contained many, the latter few bacilli; the heart's blood contained none.

It is apparent from these experiments that the death of the animals—pigeons and rabbits—is not closely bound up with the multiplication of the bacilli in the general circulation. The rabbit experiments indicate that the bacilli have no power to remain in the blood stream, and even when diminishing in numbers in the internal

organs still exert poisonous effects. Hence it may be concluded that poisons are liberated from the bacilli, but whether merely secreted or only yielded upon disintegration is not indicated by these tests. The next step therefore was to look for soluble toxic substances in the fluid cultures. For this purpose 24 hour glucose broth growths were employed.

Experiment 5.—(*a*) Rabbit; weight 1,950 gm. 8 cc. of Culture 669 b were injected intravenously. Immediately following the injection the animal became greatly excited, respiration became rapid, prostration followed, and death occurred in 7 minutes from respiratory failure.

(*b*) Rabbit; weight 1,700 gm. 9 cc. of Culture 617 d similarly injected. 14 minutes later this animal suddenly became excited, jumped from the basket, and died 7 minutes later of respiratory failure.

(*c*) Rabbit; weight 1,800 gm. 10 cc. of Culture 365 a intravenously injected. Died within 6 minutes under similar circumstances to those in (*a*) and (*b*).

In other words, an acutely fatal effect can be produced from large quantities of a broth culture injected intravenously. Less quantities (1 to 2 cc. per kilo) produce no immediate symptoms but cause death in from 6 to 24 hours. Differences in virulence only appear from the smaller doses and at once distinguish Culture 617 d as the most active. The massive doses of cultures exerted an injurious effect on the red blood corpuscles, which are destroyed in large numbers. On the other hand, the bacilli do not tend to agglutinate rapidly in the blood stream. By performing *intra vitam* agglutination tests, it was found that Culture 386 cd alone became rapidly agglutinated. This one was the least active, requiring the largest dose to cause acute death. The blood became free of the other cultures only after 4 to 8 hours. It appears then that bacillary embolism is not the probable cause of the acute lethal effects.

The toxicity of the fluid portion of the broth cultures was investigated. Since in these tests centrifugation alone was employed, pigeons could not be used, as the small number of bacilli remaining was sufficient to cause infection; hence rabbits were again injected. We may mention here that the injection of 2 to 4 cc. of the centrifuged fluid subcutaneously or intramuscularly into rabbits would sometimes lead to severe local and fatal infection. The fluid therefore possessed aggressive activity.

Experiment 6.—(*a*) Rabbit; weight 1,475 gm. Injected 10 cc. of supernatant fluid of glucose broth culture No. 617 d intravenously. Immediate collapse, air hunger, and death in 2 minutes. The red corpuscles were largely disorganized. The serum was reddish brown, and the remaining corpuscles appeared as mere shadows.

(*b*) Rabbit; weight 1,400 gm. Before injection the red corpuscles numbered 5,600,000 and the white 8,700 per c.mm. 12 m. 4 cc. of supernatant fluid of Culture 617 d were injected intravenously. 20 minutes later breathing was rapid and labored; the animal was very weak, and the red cells had fallen to 80,000 per c.mm. The white cells were unchanged. 1.20 p.m. Animal prostrate, the red cell count 84,000, and the white cells 8,300 per c.mm. 10 minutes later respiration ceased. The kidneys were dark brown in color, the bladder contained dark brown urine, and the few remaining red corpuscles were shadowy.

(*c*) Rabbit; weight 1,375 gm. Red cells 5,250,000 per c.mm. 11.50 a.m. Received 3 cc. of supernatant fluid, Culture 617 d. 12.15 p.m. Respiration accelerated, red cells 1,600,000. 3 p.m. Red cells 1,650,000. Died in night. The urine found was dark brown in color. Kidneys chocolate colored.

It would appear to be shown by these experiments that the acutely fatal effects of massive doses of the broth cultures as such or when separated in large part from the bacilli themselves are due to some body causing rapid and extensive blood destruction. Whether any other factor plays a part these experiments do not determine.

The next experiments were devised to rule out the factor of acidity. The different supernatant fluids exhibited acidities ranging from 2.5 to 4.5 per cent in terms of normal sodium hydroxide, with phenolphthalein as indicator. References in the literature point to the acid and especially the butyric acid content of cultures as responsible for the toxic effects. Two sets of tests were made (1) by neutralizing the broth with sodium hydroxide and (2) by comparing the acidity with the toxicity of different fluids.

Experiment 7.—Rabbit; weight 1,575 gm. Red cells 5,450,000. 12 m. 5 cc. of supernatant fluid of Culture 617 d neutralized with sodium hydroxide were injected intravenously. 12.35 p.m. Red cells 1,600,000. 1.50 p.m. Red cells 800,000. Respiration rapid, animal tires easily. 3 p.m. Red cells 600,000. Animal died during night. The autopsy showed the dark urine and chocolate colored kidneys as described above.

The exclusion of the acidity in the fluid may diminish somewhat the intensity of the blood destruction but does not remove it or

prevent the fatal issue. Experiments similar to this were made a number of times with consistent results. Moreover, comparison of toxic action and the degree of acidity was made, from which it was seen that acidity and lethal effects do not proceed hand in hand.

Experiment 8.—(*a*) Rabbit; weight 1,400 gm. Red cells 5,700,000 per c.mm. 2.45 p.m. 8 cc. of supernatant fluid of Culture 617 d, having an acidity of 1.9 per cent normal sodium hydroxide, were given intravenously. 4.45 p.m. Red cells 1,240,000. 5 p.m. Animal breathing rapidly; very weak. Died during night. Usual autopsy findings.

(*b*) Rabbit; weight 1,600 gm. 2 p.m. 10 cc. of supernatant fluid of Culture 386 cd, having an acidity of 4.5 per cent normal alkali, were injected intravenously. No symptoms appeared, and the red corpuscles were only slightly reduced in number. The animal still lives.

These experiments show that the acidity is not the main factor in causing either blood destruction or the fatal effects, and they are supported by tests on pigeons, examples of which follow; they indicate also that *Bacillus welchii* produces, in the test-tube at least, an active hemolysin.

Experiment 9.—(*a*) Pigeon. Red cells 4,645,000 per c.mm. 9.50 a.m. 0.5 cc. of 24 hour glucose broth culture No. 365 a injected into wing vein. 10.40 a.m. Red cells 4,700,000; no free nuclei present. 11.50 a.m. Pigeon drooping. Red cells 3,880,000, free nuclei appearing. 2 p.m. Red cells 2,432,000; many free nuclei. 3.45 p.m. Dying. Red cells 1,520,000. Immediate autopsy showed some but not numerous bacilli in blood; cytoplasm of the red cells stains weakly; blood serum reddish brown in color.

(*b*) Similar to (*a*). In 8 hours, when death occurred, the red cells had fallen from about 5,000,000 per c.mm. to 1,000,000. The autopsy findings were typical.

In other words, the injection of 0.5 cc. of a broth culture of active *Bacillus welchii* intravenously into pigeons causes extensive blood destruction and death in periods of from 6 to 8 hours. These results are now to be contrasted with pigeons in which the culture is injected into the pectoral muscles.

Experiment 10.—(*a*) Pigeon. Red cells, 4,500,000 per c.mm. 9.30 a.m. 0.5 cc. of glucose broth culture No. 365 a injected into breast muscle. 12 m. Drooping. 3.30 p.m. Red cells 4,600,000; no free nuclei. 4.45 p.m. Dying. Red cells 4,450,000. Autopsy showed local infection; no bacilli in blood.

(*b*) Similar to (*a*) except that Culture 617 d was employed. At the outset the red cell count was 4,450,000 per c.mm. 6 hours later, when the animal was dying, it was 4,435,000. The autopsy findings were characteristic.

These experiments several times repeated were always consistent. Intravenous injections of broth cultures are attended by extensive blood destruction and death; intramuscular injections of like doses cause death with equal certainty and rapidity but no blood destruction. Hence the blood destruction cannot be the determining factor of the lethal action. The essential toxic agent appears now not to be an acid and not an hemolysin. The next experiments relate to its filterability.

Up to the present the fluid cultures described were not wholly free from the bacilli. To remove the bacilli entirely, in order to test the toxicity of the sterile fluid, filtration through a Berkefeld N candle was resorted to. The first tests were made with filtrates obtained from ordinary glucose broth cultures. They indicated merely a low degree of toxicity for rabbits and pigeons.

Experiment 11.—A 24 hour glucose broth growth of Culture 617 d was employed as follows:

(*a*) Rabbit; weight 1,475 gm. 10 cc. of the supernatant fluid obtained by centrifugation for 20 minutes, not quite clear, and having an acidity of 4.2 per cent were injected intravenously. The animal had a severe convulsion and died almost immediately. The blood was extensively destroyed.

(*b*) Rabbit; weight 1,425 gm. 11 cc. of a Berkefeld filtrate, having an acidity of 3.9 per cent, were injected intravenously. No symptoms.

(*c*) Rabbit; weight 1,575 gm. 3 p.m. 10 cc. of the clear fluid obtained by centrifugation for 40 minutes and having an acidity of 4.5 per cent were injected intravenously. For an hour there was respiratory distress, which passed off. 7 p.m. Died. Kidneys chocolate colored Another part of this fluid first filtered, then injected into a normal rabbit, produced no effect.

This experiment shows that not only does filtration reduce the toxicity, but long centrifugation does also. The difference is not caused by the reduction in acidity observed in the filtered fluid, since neutralization of the centrifugate with sodium hydroxide did not affect its activity. The injection of the filtrate in amounts of 8 cc. into the breast muscles of pigeons caused temporary drooping but no local lesion or other severe effect. No distinction in action was

noted in the different cultures. The conclusion to be drawn from these experiments is that a certain kind of toxic product is developed in glucose broth cultures, but that prolonged centrifugation and filtration tend to remove it from the fluid. Since the action is so rapid, it does not seem probable that there is any actual relationship between the bacilli as such still remaining in the centrifuged fluid and the poisonous agent. The latter is not an ordinary acid and appears to be an hemolysin.

The next experiments throw an entirely different light on the toxin-producing property of *Bacillus welchii*. It is obvious that cultures in glucose broth in no way represent the conditions occurring during local infections in man and animals. Hence these were simulated in the following manner.

To plain beef infusion broth in 10 cc. quantities in test-tubes were added several fragments of sterile skeletal muscle of the pigeon or rabbit. The tubes, having been proved sterile, were inoculated with *Bacillus welchii* and overlaid with paraffin oil and enclosed in a vacuum jar from which the oxygen was exhausted. After an incubation of from 18 to 24 hours, the fluid was centrifuged and filtered through a Berkefeld N candle. The filtrate was always free of the bacilli. This product proved highly toxic for pigeons, guinea pigs, and rabbits, and, what should be emphasized, gave rise to inflammatory and other local lesions resembling closely those caused by the bacilli themselves. While Culture 617 d, the most virulent of all, yielded the most active filtrate, yet all five cultures gave toxic products. The degree and manner of the action of the toxic filtrates are indicated by the following illustrative protocols.

Experiment 12.—(*a*) Guinea pig. 3 p.m. 2 cc. of the Berkefeld filtrate of an 18 hour pigeon muscle broth culture of Culture 617 d were injected beneath the skin of the right thigh. The next morning at 8 a.m., the entire leg was swollen and the joints held stiffly; the scrotum was also edematous. The animal crouched in the corner of the cage. 3 days later the hair was loose, and the tissues were sloughing. Death occurred during the night. The autopsy showed disorganization of the muscles of the right leg and adjacent abdominal wall. Cocci, but no gas bacilli were present.

Doubtless the filtrate acted upon the skin and underlying muscles, inducing inflammation and necrosis, after which pyogenic cocci and

other bacteria entered the injured tissues and produced the sloughing. A dose of 1 cc. of the filtrate caused a similar but less severe lesion, from which the guinea pig slowly recovered after healing of the defect.

(*b*) Rabbit. 2 cc. of the same lot of toxin used in (*a*) were injected under the skin of the right thigh at 4.30 p.m. The next day the skin over the leg and the right scrotal sac was highly edematous. 5 days later, the edema subsided, leaving a dry necrotic area behind, which finally was thrown off and became healed.

The local effect, therefore, in the rabbit is similar to although less severe than that in the guinea pig. The effect in the rabbit of an intravenous injection of the same lot of toxin is to produce acute blood destruction and death. Thus a rabbit having a red cell count of 5,400,000 was given 1 cc. of the toxin at 10 a.m. At 11 a.m. the cells numbered 4,250,000; at 12.30 p.m., 2,550,000; at 4 p.m., 1,500,000; at 5 p.m., 1,000,000. Death took place during the night. The kidneys were chocolate colored and the urine dark.

(*c*) Pigeon. 12 m. 0.2 cc. of a similar toxin was injected into the right breast muscles. The next morning at 8 o'clock there was widespread edema of the injected site. 24 hours later the edema was subsiding. 3 days later the swelling had disappeared. On etherization and autopsy, an extensive necrotic focus was found in the injected pectoral muscles; the muscles of the opposite side were normal.

The local reaction of the pigeon to small doses of the toxic filtrate resembles that of the guinea pig to far larger doses. When 0.3 cc. of the filtrate was injected, the edema developed very quickly and death occurred in about 4 hours—even more quickly, therefore, than from massive bacillary infection. The injected muscle was already friable. No effect is produced in the red blood corpuscles. When, however, the filtrate is injected into the wing vein, hemolysis results. Thus a pigeon having a blood count of 4,280,000 was given 0.25 cc. of neutral filtrate at 9.30 a.m. At 10.30 a.m., the cells were unchanged in number and no free nuclei occurred. At 4.30 p.m. the cells numbered 3,725,000, and free nuclei were found. The next day at 1 p.m. the cells numbered 800,000, and there was marked air hunger. Death took place at 5 p.m.

This experiment, which was repeated several times, shows that *Bacillus welchii*, when the conditions of growth are suitable, yields toxic products of high potency. These products produce two sets of effects according to the manner of their injection into animals: (*a*) hemolysis, in which they resemble the effects arising from ordinary glucose broth cultures; (*b*) inflammation and necrosis of subcutaneous tissue and muscles, in which they resemble the effects produced by the bacilli themselves. Even moderate quantities of the toxic filtrate locally injected may also bring about rapid death of pigeons.

It is now possible to answer the question placed at the head of this section of our paper. The cause of death in *Bacillus welchii* infection is not a blood invasion of the microorganisms and not acid intoxication, but an intoxication with definite and very potent poisons produced in the growth of the bacilli in the tissues of the body. This poison is readily produced in broth in the test-tube in the presence of sterile non-denatured muscle. To obtain it in quantity only minimal quantities of glucose (0.1 per cent) should be added to the broth, and the incubation of the anaerobic cultures should not exceed 24 hours. The poison or toxin is a complex of an hemolysin and another poisonous body. The latter is the more toxic, since it may bring about death under conditions in which no blood destruction takes place.

The Toxic Product.

Thermolability.—The toxicity of the filtered fluid is destroyed by heating 30 minutes at 70°C. in sealed tubes and is greatly diminished by similar heating at 62°C. The fluid subjected to the latter treatment no longer causes death in pigeons, even when large doses are injected, although a degree of necrosis of the muscles still results. A test made to determine the point indicates also that the substances exerting the toxic effects do not dialyze through collodion membranes.

Antigenic Properties.—The next step taken was that of determining whether the toxic product would act as an antigen. Two sets of tests were made: (*a*) the setting up of active immunity of the pigeon; (*b*) the production of an antiserum in the rabbit.

The former is difficult to accomplish because of the necrosis caused even by sublethal injections into the pectoral muscles of pigeons. However, by giving three carefully graded injections at weekly intervals, the animals may be kept in fair condition. 1 week after the last injection, the pigeons bore two lethal doses of the toxic filtrate without reaction.

The latter is accomplished with less difficulty. Large male rabbits were employed. 2 cc. of a neutralized filtrate of Culture 617 d were injected beneath the skin of the inner aspect of the thigh. This was followed by edema involving the scrotum. The edema subsided in a few days, leaving the scrotal skin necrotic and dry. 10 days after the first injection a second one was given on the opposite side. The effects were the same as the first. A third injection of 3.5 cc. was given on the right side after a similar interval. No reaction followed. The rabbit was now bled and a series of neutralizations performed, as shown in Table I. The toxic filtrate was mixed with the serum from the immunized rabbit or normal rabbit and injected immediately into the pectoral muscles of the pigeon or subcutaneously into the rabbit and guinea pig.

The table shows that the blood of a rabbit which has received three injections of a toxic filtrate from a given culture is capable of neutralizing not only that particular filtrate, but the filtrate from four other cultures as well. The neutralization is effective against the filtrate obtained from several distinct cultures and for the three species of animals—pigeon, rabbit, and guinea pig—employed.

Moreover, the neutralization is not only for the toxic substance causing inflammation and necrosis of the local tissues, but also for the specific hemolysin contained in the filtrates. This is an important point, since it controverts the notion that the blood destruction results from acids produced in course of growth of the bacilli.

Experiment 13.—(*a*) Pigeon. Red cells 4,250,000; no free nuclei. 2.10 p.m. Injected into wing vein mixture of 1 cc. of toxic filtrate of No. 617 d and 1 cc. of normal rabbit serum. 3.40 p.m. Red cells 1,312,000; many free nuclei. 3.40 p.m. Death.

(*b*) Pigeon. Red cells 4,500,000. 11.45 a.m. Mixture of 1 cc. of toxic filtrate of No. 617 d and 1 cc. of immune rabbit serum injected into wing vein. 1.45 p.m. Red cells 4,264,000. 10 a.m. next day. Red cells 4,300,000. No symptoms appeared.

TABLE I.

Hr. of injection.	Animal injected.	Toxic product.		Mixed with immune or normal rabbit serum.		Local reaction.	Final result.
		Quantity.	Source.				
a.m.		*cc.*		*cc.*			
9	Pigeon.	1	617 d	0.5	Immune.	None.	Survived.
9	"	1	617 d	0.5	Normal.	In 2 hrs. extensive edema.	Died, 2.10 p.m.
9	"	3	365 a	1.0	Immune.	None.	Survived.
9	"	3	365 a	1.0	Normal.	In 3 hrs. extensive edema.	Died, 4.10 p.m.
9	"	3	669 b	1.0	Immune.	None.	Survived.
9	"	3	669 b	1.0	Normal.	In 4½ hrs. muscle greatly swollen.	Died, 1.45 p.m.
9	"	3	P-50	1.0	Immune.	None.	Survived.
9	"	3	P-50	1.0	Normal.	In 5 hrs. muscle greatly swollen.	Died, 2.30 p.m.
9	"	4	386 cd	1.0	Immune.	None.	Survived.
9	"	4	386 cd	1.0	Normal.	In 5 hrs. muscle swollen.	Died, 2.45 p.m.
10	Rabbit.	2	617 d	1.0	Immune.	None.	Survived.
10	"	2	617 d	1.0	Normal.	Extensive scrotal edema.	Recovered.
10	Guinea Pig A.	2	617 d	1.0	Immune.	None.	Survived.
10	" " B.	2	617 d	1.0	Normal.	Extensive swelling of leg and scrotum.	Necrosis. Died on 7th day.

Neutralizing Proportions.—The next experiment was designed to determine the minimal lethal dose of the toxic filtrate and the necessary neutralizing quantity of immune serum for that dose. This having been ascertained, an experiment was conducted to decide whether the neutralization took place equally in multiple proportions.

Pigeons were employed for the tests. The minimal lethal dose of the toxic filtrate employed proved to be 0.3 cc., from which death resulted in about 8 hours. The perfectly neutralizing quantity

of the immune serum for this dose was 0.2 cc. When mixed together and injected into the pectoral muscles no reaction followed. The minimal protective dose of the immune serum was much smaller; namely 0.05 cc. But with this dose considerable local reaction manifested itself.

The experiment with multiple proportions of toxic filtrate and immune serum was made with twenty-five doses of each. Hence 7.5 cc. of the toxic filtrate and 5.0 cc. of the immune serum were mixed and the entire volume was then injected into the breast muscles of each of two pigeons. No signs of intoxication developed, and aside from slight local edema in one of the pigeons, no symptoms whatever appeared. From this it was concluded that the toxic filtrate and antitoxic rabbit blood neutralized each other perfectly in multiples of the single doses. In this respect the two resemble the corresponding toxins and antitoxins of *Bacillus diphtheriæ* and *Bacillus tetani*.

Protective and Curative Properties.

The experiments described having clearly shown that the toxic products of the growth of *Bacillus welchii* exhibit antigenic activities and readily give rise to the formation of active antitoxic substances, the obvious next step was to determine whether the immune serum developed possessed protective and curative properties. Two sets of tests bearing on these questions have been made.

In one, vegetative bacilli have been injected into the breast muscles of pigeons mixed with or followed by the immune serum. The result is to prevent or reduce the pathogenic effects otherwise produced. Normal rabbit serum has no such power of control. These experiments will be published in detail later.

In the other, the object was to imitate conditions of natural infection in man with a view to preventing infection from arising. For this purpose, the bacilli were cultivated by Dunham's method so as to obtain spores. Bits of gauze were impregnated with the sporulating cultures and thrust into the breast muscle of the anesthetized pigeons with a small hemostat. The wound at once filled with blood and became sealed. Hence the conditions of a foreign body carrying active spores of the gas bacilli imbedded in muscle tissue

and protected from access of air as occurring in man were reproduced on a small scale. The test proved a severe one. Five pigeons were employed in a series. One only was treated with the immune serum, the other four serving as controls. This plan was adopted to remove the fallacy of an accidental survival of the treated animal. 2.0 cc. of the immune serum were injected, partly about the wound, partly in the opposite breast. The four inoculated but untreated pigeons developed typical local lesions and succumbed in 20 to 40 hours after inoculation. The treated animal never showed any local or general symptoms, survived, and the wound healed about the foreign body.

The experiments briefly reported in this section of the paper seem to possess considerable importance. They indicate, indeed, that in *Bacillus welchii* infection in nature the development of the spores into vegetative bacilli may be prevented by a protective inoculation of an antitoxic serum, and also that the vegetative bacilli may be deprived by such a serum of their toxic products, which now appear to be their real offensive instrument. We are confronted, therefore, not only with a new point of view regarding the manner of the pathogenic action of the Welch group of bacilli but also with a new means of combating their pathogenic effects.

DISCUSSION.

The experiments presented appear to admit of one interpretation only; namely, that the Welch bacilli, under suitable conditions of growth, produce an active exotoxin, to which their pathogenic effects are ascribable. The toxic product, moreover, acts upon the local tissues and the blood in a manner identical with the action of the cultures. With the toxic product animals may be immunized actively and yield an immune serum which neutralizes the toxin perfectly and in multiple proportion. The toxic bodies would seem to be at least two in number: one causing blood destruction, hence an hemolysin, and the other acting locally on the tissues and blood vessels, causing edema and necrosis and probably exerting general toxic action in addition. The part each plays in bringing about the lethal effect seems to be determined by the manner of inoculation: to bring out the

hemolytic action intravenous injection is indicated; to bring out the locally destructive action, subcutaneous or intramuscular injection is required.

This conception of the manner of pathogenic action of the Welch bacilli is totally different from any view previously held. It is true that others have attributed the general symptoms in *Bacillus welchii* infection to an intoxication; but the poisoning meant was one ascribed on the one hand to decomposition products of the infected tissues (E. Fraenkel) and on the other to ordinary endotoxin absorption (Metchnikoff, Korentchewsky, Kamen, Herter, Passini). Other views have also been expressed and insisted upon, and they would ascribe the locally destructive effects of the bacilli to mechanical action (Taylor) or to the production of fatty acids which also through the setting up of an acidosis bring about a lethal termination (McCampbell, Stewart and West, Wright).

Reference will be made only to the views expressed by recent writers who have encountered gaseous gangrene in connection with gun-shot wounds of the great war. Thus Weinberg,[6] who believes that the gas-producing bacilli do not cause the gangrene, but that the condition precedes the infection, has obtained toxic and antitoxic products from various anaerobic bacteria isolated from gangrenous wounds. With *Bacillus perfringens* (of the *Bacillus welchii* group) he has prepared an antibacterial serum, but he failed to detect either the exotoxin or its corresponding antiserum.

Kenneth Taylor[7] considers that gaseous gangrene is the result of the mechanical action of the gas produced in a local focus of developing saprophytic bacteria. He specifically draws the distinction between *Bacillus tetani* and *Bacillus welchii* infections, since with the former the toxin is the active factor, while with the latter the mechanical effect of the gas is paramount. The mechanical process he conceives to be as follows: *Bacillus welchii* attacks the carbohydrates of muscular tissue and produces a large volume of gas, which, being unable to escape from the tissues, exerts pressure upon the blood vessels, impeding the circulation so that necrosis results. The necrotic tissue is invaded by putrefactive bacilli which disorganize it.

Sir Almroth Wright[8] holds that *Bacillus welchii* operates through the production of an acid condition of the blood and tissues, through which the antitrypsin is diminished. Because of this diminution, tryptic digestion of the proteins is permitted and the bacilli are thus provided with a highly favorable medium of growth, so that multiplication becomes explosive in nature. The intoxication following is in fact an acidemia.

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[6] Weinberg, M., *Proc. Roy. Soc. Med.*, 1916, ix, Occas. Lect., 119.

[7] Taylor, K., *Bull. Johns Hopkins Hosp.*, 1916, xxvi, 297; *J. Path. and Bacteriol.*, 1916, xx, 384.

[8] Wright, A. E., *Proc. Roy. Soc. Med.*, 1916–17, x, Occas. Lect., 1.

Conradi and Bieling[9] distinguish two phases of action of the bacilli. In the first or fermentation phase, the carbohydrates are attacked, and lactic, butyric, propionic, and succinic acids are formed, which are the immediate causes of the edema and necrosis of the tissues. In the second or saprophytic stage, the spore-bearing organisms appear and appropriate the dead tissue, giving rise to putrefaction and consequent intoxication.

These brief extracts readily indicate not only the wide diversity of opinion held by recent students of the pathogenesis of gas bacillus infection in man, but show also how remote the conceptions are from that of a specific pathogenetic process, due to the action of particular toxic substances, which is the basis of the conviction derived from the experiments described by us. According to our view, infection by *Bacillus welchii*, like infection by *Bacillus tetani*, essentially resolves itself into an intoxication, in which an exotoxin yielded by the multiplying organisms constitutes the chief danger. The two conditions differ, however, with respect to the local effects produced on the tissues, since the tetanus toxin does not possess inflammatory and necrotizing properties. The Welch bacilli, therefore, grow more abundantly and produce wide destruction of tissue, in which process they are soon assisted by the usual pyogenic microorganisms, which quickly obtain a foothold in the disorganized structures.

SUMMARY.

Five cultures of *Bacillus welchii* have been studied and compared. Four came from infected wounds in the western theatre of war, and one was obtained from a personal article of clothing. Each culture possesses the essential characteristics ascribed to that group of bacteria.

The infectious processes caused by the five cultures in rabbits, guinea pigs, and pigeons, are local in character; and very few or no bacilli enter or are found in the general blood stream during life or immediately after death.

Glucose broth cultures, injected intravenously, are fatal to rabbits. Death occurs almost immediately or after a few hours. Agglutinative bacterial emboli have been ruled out as the cause of death,

[9] Conradi, H., and Bieling, R., *Münch. med. Woch.*, 1916, lxiii, 1608.

as has been an acid intoxication. The fluid part of the culture acts in the same manner as the full culture and irrespective of neutralization with sodium hydroxide.

The full cultures and supernatant fluid are hemolytic when injected directly into the circulation of rabbits and pigeons, and the acute death produced may be ascribed to a massive destruction of red corpuscles. The passage of the fluid portion of glucose broth cultures through Berkefeld filters reduces materially the hemolytic and poisonous effects.

Cultures of the Welch bacilli in plain broth to which sterile pigeon or rabbit muscle is added are highly toxic, and the toxicity is not noticeably diminished by Berkefeld filtration. The filtrates are hemolytic when injected intravenously and inflaming and necrotizing when injected subcutaneously and intramuscularly. The local lesions produced in the breast muscles of the pigeon closely resemble those caused by infection with the bacilli.

The toxicity of these filtrates is not affected by neutralization with sodium hydroxide, but is materially reduced by heating to 62°C. and entirely removed by heating to 70°C. for 30 minutes.

Successive injections of carefully graded doses of this toxic filtrate in pigeons and rabbits give rise to active immunity. The blood taken from the immunized rabbits is capable of neutralizing the toxic filtrate *in vivo* and *in vitro*. The filtrate has therefore been designated as toxin and the immune serum as antitoxin.

The antitoxin neutralizes the toxin in multiple proportions. Hence the latter would seem to possess the properties of an exotoxin. Moreover, it neutralizes the hemolytic as well as the locally injurious toxic constituent.

Antitoxic serum prepared from a given culture of *Bacillus welchii* is neutralizing for the toxins yielded by the other four cultures of that microorganism.

The antitoxin is protective and curative against infection with the spore and the vegetative stages of *Bacillus welchii* in pigeons. The limits of the protective and curative action are now under investigation.