TOXIC SUBSTANCES PRODUCED BY PNEUMOCOCCUS.*

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There is much obscurity concerning the relation between the growth of pneumococci in the body and the symptoms and death induced in animals and in man. The view that bacteria act mechanically was long ago held to be inadequate and incorrect. The discovery of toxic substances produced by the growth of certain bacteria outside the body, notably by *Bacillus diphtheriæ* and *Bacillus tetani,* gave support to the supposition that the effects of the growth of all bacteria in the body were due to poisons arising through the metabolic activities of the cells. Since the poisons, however, could not be demonstrated in the culture fluids of other bacteria, this supposition has not received final proof. The demonstration by Pfeiffer that certain bacteria killed by heat or other harmful agents are toxic, gave support to the view that the effects produced in the body by the growth of bacteria are not due to the products of growth of the bacteria, but to toxic substances set free upon their death and dissolution. The attempt to demonstrate these so called endotoxic substances in a large number of bacteria, however, has not been successful, and in the cases where they have been demonstrated, the exact relationship of the poisons to the symptoms and death induced has not yet been rendered perfectly clear.

These theories, however, do not exhaust the conceivable ways in which the growth of bacteria within the body may exert harmful effects, and it is possible that the effects may not be due to substances which may be isolated, or even be detected by injection into the animal body.

Nevertheless, at the present time, the only hope of arriving more closely at a conception of the process seems to be in the iso-

* Received for publication, August 1, 1912.

lation, from the bacteria or their cultures, of substances having a similar effect to that of the living bacteria themselves.

Thinking that possibly such substances might be present in excess in the fluids of the infected animal, a series of experiments, of which the following is typical, were undertaken.

A rabbit was inoculated with an overwhelming dose of virulent pneumococci. Just before death, the animal was bled to as near complete exsanguination as possible, the serum was removed and passed through a Berkefeld filter in order to remove the bacteria, and as quickly as possible this filtered serum was injected into the veins of a normal animal.

The following is a protocol of one of these experiments:

Rabbit 7.—Weight 2,870 gm. Temperature 39° C.

Feb. 9, 1912, 12:05 P.M. Injected intravenously an emulsion in salt solution of the bacteria obtained by centrifugalization from 1,000 c.c. of a 24 hour bouillon culture of pneumococcus I.4[2].

12:15 P.M. Bowels move actively. 1:10 P.M. Temperature 40° C. 2:10 P.M. Temperature 39.8° C. 3.10 P.M. Temperature 39° C. 4:10 P.M. Temperature 38.4° C. 5:00 P.M. Animal moribund. Animal bled as completely as possible. Blood allowed to clot, serum obtained by centrifugalization. Serum passed through Berkefeld filter under pressure.

Rabbit 8.—Weight 1,600 gm. Temperature 38.2° C.

Feb. 9, 6:30 P.M. Injected into ear vein filtered serum obtained from rabbit 7.

10:30 P.M. Temperature 39.6° C. Animal shows no symptoms.

Feb. 10, 10:00 A.M. Temperature 39° C. Weight 1,600 gm. Animal appears well. 6:00 P.M. Temperature 39° C.

Feb. 11, 10:00 A.M. Weight 1,620 gm.

During the succeeding days a record was kept of the weight. It lost slightly, weighing on February 24 1,510 gm. No other changes were noted.

Other similar experiments were conducted on a series of rabbits, but in no case were acute toxic symptoms noted, nor were chronic changes, as determined by loss of weight, observed. It therefore became evident that if the symptoms of infection by pneumococcus are due to soluble toxins formed in the serum of the infected animal, they are fixed by the tissues as rapidly as formed, or are extremely labile and so lose their toxicity on removal from the body, or possibly are held back with certain of the proteins during filtration.

Attention was then directed to the production of toxic substances by the action of immune serum and complement on pneumococci.

Friedberger,[1] in his study of the nature of anaphylaxis, has shown that by

[1] Friedberger, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 692; 1909-10, iv, 636.

the action of complement on the precipitate formed by the addition of immune serum to the serum antigen, a toxic substance is formed which, on injection into a guinea pig, produces acute symptoms and death, apparently identical with the symptoms and death occurring in anaphylaxis due to the second injection of egg albumin or serum. To the toxic substance contained in the albumin-immune-serum-complement mixture, Friedberger gave the name anaphylatoxin. He considered that this experiment gave the final proof of the antibody reaction in anaphylaxis. Later he[2] showed that a similar toxic substance appeared when bacteria were treated with immune serum and complement outside the body. This "Bakterien-anaphylatoxin" he considered identical with the anaphylatoxin produced from serum and identical with the substances formed within the body on the second injection of protein, to which hypothetical substances he considers the symptoms due. Friedberger thinks the symptoms in infection are due to this substance set free in the body. He defines infection as a "mild, protracted form of anaphylaxis" and anaphylaxis as an "extreme, acute form of infection." Therefore it is not necessary to assume any specific bacterial toxin in any infection, but Friedberger thinks in all infections the symptoms are induced by the same poisonous substance, and the different manifestations of the different infections depend only on different modes of infection, localization, etc., of the different microörganisms.

Neufeld and Dold[3] were able to repeat the experiment of Friedberger, using pneumococcus as antigen. We have also repeated this experiment with pneumococcus. Since no essential differences in the results from those of Neufeld and Dold and of Rosenow[4] were found, the protocols of the experiments are not given.

At least three different views as to the origin of the toxic substance in anaphylaxis have been advanced. Vaughan and Wheeler[5] first showed that by the action on protein of alkali in boiling alcohol, substances were formed which, when injected into animals, produced acute toxic symptoms and death. They suggested that anaphylaxis is due to an analogous splitting of the protein, in this case the splitting of the injected protein occurring in the body as a result of the action of ferments or antibodies which have developed as an immunity response to the first injection.

On the other hand, it has been maintained by Nicolle, Weichhardt, Wolff-Eisner and others that the facts may be best explained by assuming that the antibody reaction does not result in a splitting of the protein, but in the case of bacteria, at least, in a lytic action, resulting in the setting free of preformed toxic substances contained within the bodies of the bacteria, endotoxins.

Finally, Wassermann and Keysser[6] hold that the toxic substance does not arise from the antigen, but is a product of the ambocepter of the serum. They

[2] Friedberger, E., and Vallardi, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 94.

[3] Neufeld, F., and Dold, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 55.

[4] Rosenow, E. C., *Jour. Infect. Dis.*, 1911, ix, 190.

[5] Vaughan, V. C., and Wheeler, S. M., *Jour. Infect. Dis.*, 1907, iv, 476.

[6] Keysser, Fr., and Wassermann, M., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1911, lxviii, 535.

think that the so called anaphylatoxin may be produced by the interaction of inactive substances, as *kieselguhr,* with immune serum, in this case the inactive substance taking the place of the bacterial antigen.

It is impossible here to review all the evidence bearing on this subject, but the experiments to be detailed below will be considered as having a bearing on these various theories.

Neufeld and Dold[7] showed that in order to produce similar toxic substances from bacteria, the presence of serum is unnecessary, but that by simple extraction of bacteria in salt solution containing 0.1 per cent. of lecithin, such toxic substances could be obtained. Rosenow[8] then showed that if pneumococci are merely placed in salt solution and allowed to remain in this solution for forty-eight hours at 37° C., the extract so formed is toxic and on injection intravenously into guinea pigs, symptoms and acute death, like that seen in serum anaphylaxis, result. The nature of the change occurring in the bacteria which renders them toxic has been considered by Rosenow to be a true autolysis. He thinks that, owing to the activity of the ferments contained in the bacterial cell, self digestion occurs and the products of this protein digestion are toxic. He thinks an identical or analogous change occurs within the body of the infected host. Upon the property of self digestion, according to him, depends the virulence of the infecting organism. If the organism autolyzes readily, it is therefore virulent; if it autolyzes only slightly, little of the poison is set free and the organism possesses slight virulence.

Since the symptoms produced by the injection of anaphylatoxin or bacterial extracts resemble very remotely those occurring during the pneumococcus infection, it is not certain that this poison has any relationship with the symptoms of the natural infection. Nevertheless, the production of a toxic substance from the bodies of pneumococci is of great importance since, as is well known, the bodies of pneumococci killed by heat are slightly toxic under ordinary circumstances, and large amounts may be injected into susceptible animals, namely, the rabbit and guinea pig, without producing marked effects. This poisonous extract may be of great importance in the pathogenesis of the disease, but in order to establish this, it will first be necessary to learn more concerning the nature of the substance and how it is produced.

Hence experiments were undertaken to confirm or disprove the observations of others as to the main fact, and secondly, by modification of the conditions, to learn how toxic extracts may uniformly be prepared, in order that they may be studied further.

[7] Neufeld, F., and Dold, H., *Berl. klin. Wchnschr.,* 1911, xlviii, 1069.
[8] Rosenow, E. C., *Jour. Infect. Dis., loc. cit.*

EXPERIMENTS TO DETERMINE THE TOXICITY FOR GUINEA PIGS OF EXTRACTS OBTAINED BY EXTRACTION OF PNEUMOCOCCI IN SALT SOLUTION.

The first set of experiments consisted in determining the toxicity of extracts obtained by keeping our stock organism, designated as N–I, in salt solution for various lengths of time. This organism was obtained from Professor Neufeld and was originally isolated from the sputum of a patient with pneumonia. It is the one which has been largely employed by him in his experiments and in the production of his immune serum. It has been frequently passed through mice and possesses high virulence for these animals, 0.000001 of a cubic centimeter of a bouillon culture uniformly killing one of these animals in less than twenty-four hours. It readily retains its virulence on artificial culture medium, and for this reason it is especially suitable for experimental work and has therefore been largely employed in our laboratory. It is a typical pneumococcus, autolyzes readily in salt solution and is easily soluble in bile. The cultures used in this set of experiments were, in most instances, the first or second passages from mice, though in some cases they had been on artificial medium for as many as six transfers.

In all these tests the organisms were grown in bouillon and were removed from the medium by centrifugalization in order to make sure that we were dealing only with the bodies of the bacteria and not with substances derived from the culture medium. If solid medium is employed, it is almost impossible to obtain the bacteria free from small particles of the medium which may not only furnish toxic substances, but, unless the extract be carefully centrifugalized, when injected into the circulation of animals, may cause death by mechanical stoppage of vessels. The objection to the use of solid medium is especially important in the case of blood or serum agar. The presence of small amounts of the complex blood or serum in the mixture renders the analysis of the phenomenon difficult since it cannot be stated with certainty that the effects obtained are due primarily to pneumococci. Wassermann and others have claimed that shock and death like that seen in anaphylaxis may be due to the injection of foreign serum alone.

The culture medium employed in these experiments was a plain bouillon reaction, approximately + 0.6 to phenolphthalein. In this medium pneumococci grow well, causing a dense uniform clouding of the medium in eighteen to twenty-four hours. At this time the medium still reacts faintly alkaline to litmus. The number of bacteria per cubic centimeter in a twenty-four hour culture of N–I is usually between 250,000,000 and 270,000,000. Since this number was determined several times, the number of bacteria injected is usually designated as those contained in a given number of cubic centimeters of the culture medium. From this a fair idea of the actual number of bacteria injected may then be calculated.

The bacterial bodies having been sedimented by centrifugalization, the supernatant fluid is carefully and completely pipetted away. In the first experiments the salt solution was added directly to this sediment and an emulsion was made by means of a pipette. In all the later experiments, the bacterial bodies were washed in 0.85 per cent. salt solution and again thrown down in the centrifuge and the emulsion made from these washed bacteria. It was found by experiment that moderate amounts of the medium alone may produce considerable effects in the injected animal,—even death, if larger amounts are employed. The symptoms are probably caused by peptone. It is extremely important in experiments of this kind that effects are not ascribed to toxic substances derived from the bacteria, when they are really due to peptone or other substances contained in the medium. After adding the desired amount of salt solution to the bacterial sediment, a uniform emulsion was made by means of a pipette and this emulsion, in a loosely stoppered bottle, was placed in a thermostat or on ice for the desired length of time.

In certain of the experiments the emulsion was covered by a layer of ether, as advised by Rosenow, but in the larger number this was not done. That lysis of the bacteria goes on more rapidly or completely when ether is added was not confirmed. Moreover, what is more important, the injected animals may show marked effects due to the ether alone.

Various concentrations of bacteria in the salt solution used for the extract were employed, the same culture being employed in making extracts of various concentrations. In most of the extracts, 1 c.c. represented the bacteria from 10 to 20 c.c. of bouillon culture, but extracts of lesser and greater concentration were also employed. Usually 6 to 8 c.c. of the extract were injected, but as much as 10 c.c. and as little as 1 c.c. were also inoculated. The extracts injected represented the bacterial bodies obtained from as little as 6 c.c. to as much as 180 c.c. of bouillon culture, or 1.5 billion to 45 billion bacteria. Most tests were made with doses of extract representing about 10 to 20 billion bacteria. After standing in the incubator for twenty-four hours, the fluid usually clears and the bacteria fail to stain entirely by the Gram method, and stain faintly or not at all with the ordinary dyes. Frequently even after autolysis is said to be complete, the forms of the bacteria in the centrifugalized sediment may still be made out when the preparation is stained with the Manson stain. Most extracts were allowed to remain at 37° C. for from twenty-four to forty-eight hours. Extracts were tested that had been kept at 37° C. for as short a time as eighteen hours and as long as six days. Extracts kept on ice from

twenty-four hours to as long as twenty-five days were tested also. Again extracts were used that had been kept at 37° C. for from twenty-four to forty-eight hours and then for various lengths of time on ice. No special effort was made to determine the extent of lysis by a determination of soluble nitrogen, or by determining the changes produced in the rotation of polarized light by the solutions. The tests we have made with the polariscopic method have shown that if changes occur, they are so slight as to be negligible. Certain of the changes recorded by others and on which stress has been laid, have been so slight as to be within the limits of error of the best instruments. The changes undergone by the emulsion are by no means entirely proteolytic, and to employ this method, under the given conditions, as an indication of proteolytic cleavage, is a doubtful expedient.

Guinea pigs were used in making the tests in this set of experiments. At first the guinea pigs employed weighed 250 to 340 gm., but in all the later experiments, the animals were smaller, weighing between 200 and 250 gm. The injections were made into the external jugular vein, and usually 4 to 8 c.c. of fluid were injected, but in a few instances as much as 10 c.c. were injected. Of 144 animals 5 showed immediate symptoms following the injection which could be clearly ascribed to the incomplete removal of ether from the extract. In 90 there were no symptoms at all, or symptoms so slight that it did not seem justifiable to consider them as representing more than the shock due to the operation and injection of the considerable amount of fluid. Following the injection certain animals showed some tremor, the hair becoming ruffled and the animal appearing quiet and sick. In 26 instances the symptoms were described as of moderate severity and could possibly be considered analogous to the mild shock seen in anaphylaxis. The animals frequently coughed, had somewhat labored breathing, and marked tremor. In only 23 animals, however, were symptoms seen which could be definitely considered as resembling those seen in true anaphylactic shock. In most of these the symptoms very closely resembled those seen in true anaphylaxis. The animals coughed, voided urine, had marked dyspnea, and in many cases convulsions.

Seven of these animals died acutely within ten minutes. Where autopsy was performed quickly, the heart was found still beating and the lungs were usually voluminous, though in only one instance did the lungs remain distended, taking the shape of the chest and being firm and dry, as seen in typical anaphylactic death. Small focal hemorrhages were seen in the lungs and in two cases subepicardial hemorrhage.

In the seven instances the symptoms and autopsy findings may therefore be said to resemble closely those seen in anaphylaxis, and if occurring in guinea pigs following the second injection of protein, would undoubtedly have been considered as typical anaphylactic shock.

Of the other 16 animals showing marked symptoms, 10 recovered. Of the remaining 6, 1 died in one and a half hours, 1 in three and a half hours, 1 in twenty-four hours, and the others in from eight to twelve hours. In addition

to the 5 animals which died within twelve hours, there were 51 animals which showed no, or only moderately acute, symptoms, but which died within twelve hours. These 56 animals presented fairly characteristic symptoms, and at autopsy showed lesions such as have been described as occurring in late anaphylactic death. Even where no marked acute symptoms arose, they all had some dyspnea and coughing and appeared quite sick within an hour of the injection, the hair being ruffled and marked tremors occurring. Many passed bloody urine, others showed a slight bloody frothy discharge from the nostrils; and after periods varying from one and a half to twelve hours they died, frequently in convulsions. At autopsy the peritoneum contained a very little blood-tinged fluid or large amounts of clotted blood. The bladder was frequently distended with dark bloody urine. Focal hemorrhages existed in the wall of the stomach and intestine. Similar hemorrhages occurred in the intestine, mainly about the cecum and adjacent portions but sometimes throughout. The lungs were moderately distended and usually dry, although sometimes edematous, and focal hemorrhages, from pin-point size up to 2 to 3 mm. in diameter, were scattered throughout almost constantly. Frequently there were well marked epicardial hemorrhages. Cultures and films made from the heart's blood showed in some cases the presence of a moderate number of organisms; in others the cultures were sterile. That the symptoms and death were not due to infection was rendered most probable by the fact that in many cases no organisms could be demonstrated and also because in guinea pigs, no matter how severe the degree of infection, death rarely occurs within twelve hours. Moreover, the pneumococci in this series of experiments were not very virulent for guinea pigs. Undoubtedly in many of the extracts living organisms were present, but the fact that 71 out of the 144 animals recovered, many of them having received large injections of concentrated extracts, shows that the number of living virulent organisms in the extracts was comparatively small. It would seem, then, that these 56 animals died of intoxication induced by substances contained in the extract.

In the report by Dold dealing with the intoxication of guinea pigs by extracts of pneumococci in lecithin containing salt solution, it is noted that most of the animals did not die acutely, but in from one to six hours. Notwithstanding this late death, they are considered to have shown the features of anaphylactic death. Undoubtedly the kind of intoxication and death occurring in the fifty-six animals described in our series corresponds to that described by Dold.

An attempt to bring the symptoms and kind of death into relation with any of the variables in the preparation of the extract or mode of injection has been fruitless. Some of the animals dying acutely were injected with extracts of low concentration, and others with extracts of high concentration. The same is true of the animals dying late. Nor did the period of autolysis seem to have any

effect on the result. One of the animals dying acutely had been injected with extract kept forty-eight hours at 37° C., and four days on ice; another with extract kept forty-eight hours at 37° C., and five days on ice; another with extract kept forty-two hours at 37° C., and four days on ice; two others with extracts kept forty-eight hours at 37° C., and the remaining two with extracts kept for twenty-four hours at 37° C. Frequently, it was impossible to reproduce the phenomena, even though the conditions were exactly identical so far as could be determined. In two of the animals dying acutely, a few drops of blood were allowed to flow into the syringe and mix with the extract in the syringe before injection. It was thought that some reaction might have taken place between the small amount of blood and the extract. This procedure, however, was repeated in eight other guinea pigs without producing acute death in any case. In one instance, one cubic centimeter of fresh blood was added to the extract before injection. Seven guinea pigs were injected with extracts to which had been added various amounts of fresh guinea pig complement, and the mixture had been kept at 37° C. for from one to two hours. Acute death occurred in only one of these animals. This was quite like that occurring in anaphylactic shock and occurred in an animal which had been injected with three cubic centimeters of extract plus four cubic centimeters of complement, and the mixture kept at 37° C. for two hours.

The result of this set of experiments was therefore inconclusive, in so far as finding a means of producing an extract that would uniformly kill guinea pigs with symptoms like those seen in anaphylaxis. Although seven animals died acutely presenting symptoms resembling acute anaphylactic death, and fifty-six died within twelve hours presenting features resembling those seen in the more chronic forms of anaphylactic death, the results were not constant and striking enough to permit a determination of the exact conditions under which extracts producing acute death may be constantly obtained.

It was thought that possibly the failure to obtain more constant and striking results might be due to the race of organism studied, and consequently a series of tests was made with extracts prepared

from two other races. Both the organisms were obtained from pneumonic patients and had high virulence for mice. The extracts were tested on forty guinea pigs, none of which died acutely. Eight showed moderate or severe symptoms following the injection, and eighteen died within twelve hours. At autopsy these showed conditions resembling those previously described as present in the animals dying late.

Extracts were also prepared from cultures of pneumococci made in bouillon directly from the blood of pneumonia patients. Two extracts made from two races of pneumococci (Davis O′ and Baron O′) were tested, but the results did not differ from those previously mentioned.

Extracts were also prepared from two cultures kindly sent us by Dr. Rosenow. The following is a brief report of the results obtained with them.

Culture 622^{4} (*R–I*).—Blood agar. Received Dec. 12, 1911. This organism was "isolated from the blood on the second day of the disease in a case of lobar pneumonia, and has been passed through four guinea pigs."

Three extracts, prepared as in previous experiments, were tested on four guinea pigs. None showed marked symptoms. One died within twelve hours and showed the lesions previously described.

Culture 602^{31} (*R–II*).—Blood agar. Received Dec. 12, 1911. This organism was "originally isolated from consolidated lung after death and has been passed through thirty-one guinea pigs."

Two extracts were prepared in the usual way and tested on four guinea pigs. None showed marked symptoms. All recovered.

A test of the virulence of this organism (602^{31}) showed that while it had high virulence for mice, its virulence for guinea pigs, in spite of the thirty-one passages, was not very high. An attempt was therefore made to raise its virulence for guinea pigs before retesting the extract. The pneumococci were passed through a series of guinea pigs, inoculating directly from the abdominal cavity of one guinea pig to that of the next. After passage through twelve guinea pigs in this way, the virulence had been somewhat increased.

Cultures were made at various times from the peritoneal cavity of the dead guinea pigs and extracts were made from the cultures. The extracts were made from cultures of the first, sixth, seventh, twelfth, and thirteenth passages, in plain broth, and in 2, 6, and 8 per cent. glucose broth. All were covered with ether during autolysis. The extracts were tested on sixteen guinea pigs, none of

which died acutely, and which showed symptoms like those seen in anaphylaxis. Eight guinea pigs showed practically no early symptoms whatever. Three showed quite marked immediate symptoms of collapse, which seemed to be associated with incomplete removal of the ether. Only two showed marked symptoms which were like those seen in acute anaphylaxis. Six recovered. Eight died within twelve hours showing symptoms and autopsy findings like those seen in the similar group with other extracts. One died in twenty-four hours.

Hence, so far as tested, these extracts did not differ materially in their effect from those prepared from the cultures which were less virulent for guinea pigs.

The virulence for guinea pigs of the N–I culture used in the previous experiments was low. While 0.000001 of a cubic centimeter of the twenty-four hour culture was uniformly fatal for mice within twenty-four hours, as large quantities as the bacteria from five cubic centimeters of culture did not kill guinea pigs uniformly. Of six guinea pigs injected with 1.1 cubic centimeters, four recovered and two only died after eight days.

An effort was made to increase the virulence of this race for guinea pigs in order to determine whether the inconclusive results might be due to the low virulence for these animals. The same method was employed to raise the virulence of this organism as that used in the case of organism R–II. After passing through the abdominal cavities of fourteen guinea pigs, a culture was made in 6 per cent. glucose bouillon and an extract prepared. This was tested in five guinea pigs. One showed marked symptoms and died acutely, in a manner resembling acute anaphylactic death. The others recovered.

RESULTS OF THE STUDY OF SALT SOLUTION EXTRACTS.

As compared with the reports of others, the results of the study of the toxicity of extracts of pneumococci in salt solution have been disappointing. The observations of Dold would not lead one to expect to find the extracts acutely toxic with any great regularity, while those of Rosenow would indicate that acutely toxic extracts are obtained without difficulty. Of the 213 guinea pigs we injected with salt solution extracts of the several races of pneumococci, only eight died acutely. In these the symptoms present and the autopsy

findings resembled those present in anaphylaxis. Eighty-three of the animals died in from one to twelve hours under fairly characteristic conditions, such as are sometimes seen in sensitized animals dying several hours following the second injection of a protein. So far we have not found the exact conditions upon which the development of toxicity in the salt solution extracts depends. It is, of course, possible that the varieties of guinea pigs are responsible for the irregular results. Rosenow[9] states in his last communication that the susceptibility of guinea pigs to the action of extracts is greatly increased by starvation. We have made no changes in the regular feeding of the animals tested. Further study may show that factors which we have overlooked in the preparation of the medium or mode of injection, are responsible for the failures, but without a knowledge of these factors any quantitative study of the problem will be impossible.

STUDY OF PERITONEAL WASHINGS.

Since the salt extracts of pneumococci did not show as high toxicity as was anticipated, it was held possible that in the peritoneal cavity the solution of the bacteria might go on at a more rapid rate, from which cavity solutions might be obtained of greater and more constant toxicity.

Although the experiments on rabbits, mentioned early in the paper, to determine the toxicity of the blood of infected rabbits proved negative, yet it seemed possible that in the case of peritoneal infection, the toxin might not be so quickly fixed, or might be formed in such overwhelming amount that it might be found later. Friedberger and Nathan[10] have reported finding "anaphylatoxin" in the peritoneal cavities of guinea pigs into which *Bacillus prodigiosus* had been injected. Rosenow also states that the peritoneal exudates following the injection of pneumococci are toxic.

The guinea pigs from which the exudate was obtained were infected by intraperitoneal injection. As soon as possible after death the peritoneal cavity was opened and washed out with a small amount of normal salt solution.

[9] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, xi, 94.

[10] Friedberger, E., and Nathan, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1911, ix, 444.

Usually 10 to 15 c.c. sufficed to obtain sufficient fluid for the subsequent injection. After mixing thoroughly with the small amount of exudate usually present, the fluid was removed by a pipette and placed in a powerful centrifuge in which the cells and the larger number of bacteria were removed. The clear fluid was injected into the jugular vein of the animals used in the test. Since it was possible that the pneumococcus which had grown in the animal might be more susceptible to lysis or yield toxin more readily than those grown on artificial media, the sediment obtained on centrifugalization of the peritoneal washings, consisting of bacteria and a varying number of cells, had added to it 10 to 15 c.c. of salt solution and the resulting emulsion was placed at 37° C. for twenty-four to forty-eight hours, after which it was again centrifuged and the supernatant fluid injected into the vein of guinea pigs. Peritoneal washings were obtained from nine guinea pigs which had been infected with pneumococcus R–II. In two instances sufficient washings were obtained for testing each on two guinea pigs, so that in all eleven guinea pigs were injected.

Of these eleven animals, eight showed immediate symptoms like those seen in anaphylaxis, two showed but slight symptoms following the injection. One animal died during the injection. In this case the fluid was thick and viscid and death may have been due to mechanical causes. Of the remaining ten animals, four died within a few minutes with typical features of anaphylactic death and with characteristic autopsy findings. The other six all died in less than twelve hours, and at autopsy, findings, previously described in other animals dying subacutely, were found. That infection was not primarily the cause of death in these animals is shown by the fact that cultures and smears from the heart's blood were usually negative. Of course most of them would undoubtedly have died later from infection, but it seems probable that in these instances death was due to toxic substances transferred from the previously infected animals.

Seven animals were injected with the extract obtained by allowing the sediment to undergo autolysis in salt solution, as previously noted. The results were not nearly so striking as those in which the peritoneal washings were immediately employed.

The direct injection of peritoneal washings into the veins of other guinea pigs gave results which were important and striking. That four out of the ten animals had acute shock and death simulating that seen in anaphylaxis indicates that the development of the toxic substance must be much more constant in the abdominal cavity of the guinea pig than in the test-tube, if we assume that the same

process is active in the two cases. However, in the animal body the conditions are much more complex. In order to obtain an active extract under as simple conditions as possible, further efforts were directed to prepare it outside the body.

STUDY OF CHLOROFORM EXTRACTS.

The toxic effect of pneumococcus is not necessarily due to digestion products of the bacteria, as has been considered to be the case, but may be caused by preformed substances set free on the disintegration of the bodies of the bacteria, the essential process being, therefore, a form of plasmolysis, rather than of autolysis. Various methods were employed in an endeavor to throw the bacterial bodies into solution rapidly under conditions in which autolysis could, with reasonable certainty, be excluded.

Of the procedures, the only one giving any degree of success consisted in the use of chloroform, by which method we were able to obtain fairly constant solutions of the bacterial bodies. The method, however, is troublesome. The mixtures of chloroform and emulsion do not always become clear, and the injection of the solution into the veins of animals did not give constant results.

STUDY OF BILE EXTRACTS.

We next studied the solution of pneumococci obtained by means of bile. It was first shown by Neufeld[11] that pneumococci, when treated by bile or solutions of bile salts, readily undergo solution, and the emulsion, which was previously cloudy and opaque, becomes clear and translucent and on staining, the form of the bacterial bodies can no longer be made out. Although Rosenow states that ". . . complete lysis of pneumococci in weak solutions of bile salts (0.5 per cent.) . . . interferes with the production of anaphylatoxic substance . . .," preliminary experiments performed by us showed that solutions of pneumococci in bile salts, when injected intravenously, were toxic for guinea pigs. In making the solutions, a 2 per cent. solution of sodium cholate in normal salt solution was employed. It was found, however, that with this low dilution of the

[11] Neufeld, F., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1900, xxxiv, 454.

bile salt, clearing of various solutions did not always occur with equal rapidity, some solutions clearing more rapidly and others not at all.

An experiment was therefore undertaken to determine the effect of dilutions of sodium cholate on the lysis, the number of bacteria and the amount of sodium cholate being kept constant.

Two sets of tubes were prepared, one set kept at 37° C., the other on ice. It was found that the action of the cholate depends on the concentration of the sodium cholate in the emulsion, rather than on the relation of the amount of the salt to the number of bacteria present. 0.1 c.c. of a 2 per cent. sodium cholate solution was found to be much more efficient in producing solution of the given number of bacteria, when they were in a 1 c.c. emulsion, than even 0.2 c.c. when the bacteria were made into a 10 c.c. emulsion. It was found also that while lysis occurs somewhat better at 37° C., yet complete solution, when the proper mixtures are employed, may be obtained when the entire process is carried out on ice.

Even bearing in mind the above fact, however, solution of pneumococci could not always be readily obtained, even when the proper mixtures were employed. It was found that with bacteria grown in glucose bouillon, even when washed in salt solution, solution did not readily occur. It was found that when pneumococci were grown in plain bouillon and centrifugalized, then washed with the supernatant fluid from the glucose bouillon culture, the organisms, which otherwise readily dissolved, were now insoluble. It was therefore evident that the insolubility was due to something in the glucose bouillon fluid which adhered to or was absorbed by the bacteria. It seemed probable that this was acid, and this was found to be the case. Very small amounts of acid in the emulsion prevent solution of the bacteria, probably owing to the fact that cholic acid, which is insoluble, is set free.

A test was therefore made to determine the optimum reaction for lysis with bile. As is well known, by mixing primary and secondary sodium phosphate, solutions may be obtained which retain to a considerable degree their original reaction, in spite of changes occurring in the medium which would otherwise influence the reaction. By adding mixtures of primary and secondary phosphates in various proportions to the tubes containing bacteria and bile, it was found that lysis goes on most rapidly when mixtures of one

part primary to seven to eight parts secondary phosphate are employed. This gives about the reaction of blood serum.

EXPERIMENTS WITH GUINEA PIGS.

Sixty-three guinea pigs, weighing 200 to 250 grams each were injected with various amounts of the cholate extracts of pneumococci. Forty-nine showed marked symptoms similar to those seen in acute anaphylaxis, one showed moderately severe symptoms, and thirteen showed only slight symptoms or none at all. Of the sixty-three animals, thirty-seven died acutely under conditions resembling those seen in acute anaphylaxis, fifteen died in from one half to twelve hours and showed hemorrhages and other features previously described as occurring in animals dying after a few hours, one animal died during the injection,—the cause of death could not be determined,—while ten recovered. It will thus be seen that a large proportion of the animals died acutely in the manner seen in acute anaphylaxis, far more deaths occurring than with extracts prepared by extraction in salt solution. It must be remembered that these results were obtained by employing extracts prepared in various ways. The results would be much more striking if they included only animals injected with extracts prepared in the manner which we now know is most efficient for producing a toxic effect.

In the above series, the extracts injected contained only small amounts of sodium cholate. One animal dying acutely received but 0.04 of a cubic centimeter of the cholate solution. All but seven of the animals received less than 0.2 of a cubic centimeter of the 2 per cent. solution. Six received between 0.2 and 0.3 of a cubic centimeter, but of these only three died acutely. One received more than this, but it died only after four and a half hours. That the symptoms and death are not dependent upon the sodium cholate was shown, moreover, by control tests in which animals were injected with different amounts of the sodium cholate solution. One received 0.2, one 0.4, and one 0.6 of a cubic centimeter of the cholate solution in eight cubic centimeters of sodium chlorid solution. None of them showed symptoms and none died. It is quite probable that very much larger amounts could be injected without producing symptoms. It was thought possible that the reaction

might be due to some non-specific combination between the protein and the sodium cholate, of the nature of a physical reaction. But a guinea pig injected with a solution of egg albumin treated with sodium cholate solution showed no symptoms.

The extracts injected contained the substance of varying amounts of bacteria. The race of pneumococcus described as N–I was exclusively employed. After centrifugalizing from the bouillon culture, the pneumococci were washed in salt solution. Some of the extracts were prepared from quite large numbers of bacteria,—as many as those obtained from centrifugalization of 160 c.c. of the culture. The larger number, however, were prepared from the bacteria obtained by centrifugalization of 60 to 80 c.c. of the culture. Positive results were obtained also from smaller amounts. In one instance, acute symptoms and death developed after the injection of an extract prepared from the bacteria obtained from only 25 c.c. of the culture.

The solution of the bacteria was obtained by leaving the mixture on ice or at 37° C. for various lengths of time, usually from one half to several hours. In three instances acute symptoms and death were seen after the injection of extracts that had been prepared in the cold and kept on ice for only thirty minutes before injection. Three positive results were obtained with extracts that had been kept at 37° C. only ten minutes, and in five cases the mixture had been kept only fifteen minutes at 37° C. It is thus seen that the reaction which occurs between the bile salt and the bacteria occurs very rapidly, only sufficient time being required to bring the bacterial bodies into solution. Since by experiment we have learned how to produce this result most rapidly, it has been found that positive results may be obtained almost invariably, by the injection of 6 c.c. of an extract prepared from the bacteria in 50 to 60 c.c. of culture, the mixture having been kept half an hour at 37° C. The best method we have found is the following: 1,000 c.c. of a twenty-four hour culture are centrifugalized, the bacteria are washed once in salt solution, and after the second centrifugalization, the supernatant fluid is removed as completely as possible. Five c.c. of salt solution are now added, and 5 c.c. of a mixture of primary and secondary sodium phosphate solutions in the proportion of 1 to 8; a thick emulsion is thus produced, after which 2 c.c. of the 2 per cent. sodium cholate solution are added and mixed, and the mixture is placed in the water bath at 37° C. for half an hour. Sufficient salt solution is then added to bring the total up to 100 c.c., and after thorough mixing, the extract is ready for injection. The extract need not be centrifugalized unless it contains foreign particles. Six c.c. of such an extract contains the bacterial substance from the bacteria contained in 60 c.c. of the bouillon culture and 0.12 c.c. of the sodium cholate solution.

The symptoms produced and the results obtained are proportionate to the amount of extract injected, though there appear to be some individual differences in sensitiveness in the different guinea pigs. Yet two extracts prepared exactly alike do not necessarily have identical toxicity, a fact depending in part upon the varying

number of bacteria contained in different cultures. Each extract, therefore, has to be independently standardized.

TESTS OF THE BILE EXTRACTS IN RABBITS.

The toxicity of the bile extracts of pneumococci has been further studied by injection into rabbits. Rabbits weighing 700 to 1,200 grams were employed, and the injections were made into the ear veins. A considerable number of the rabbits injected with fifteen to twenty cubic centimeters of the extract died acutely in from two or three to thirty minutes. Of twenty-nine rabbits injected, eleven died acutely, eleven died in from one to twelve hours, and seven recovered or died of infection in from one to three days.

A rabbit dying within a few minutes showed, usually, the following symptoms: After the injection it would be quiet for a few minutes, then would suddenly start to run violently across the floor during which process it usually fell, and frequently would throw itself about on the floor. While on its side it would continue to make running motions with its legs, its head would be drawn back, its eyes prominent, the pupils usually dilated, the animal would cry out, make a few convulsive movements, and then died. When the symptoms were a little less acute, the progression of the disturbance could be better followed. The following protocol describes fairly well what occurred in a typical experiment:

Rabbit 5 E.—Weight 700 gm.

April 5. Injected into the ear vein 15 c.c. of an extract made by treating the bacteria from 200 c.c. of a bouillon culture with 0.7 c.c. of a 2 per cent. solution of sodium cholate. The mixture was kept on ice for thirty minutes before injection. Injection was made at 10:56 A.M. At 10:58 the animal appeared drowsy and showed some muscular weakness. 10:59. Respirations are slower; animal appears sick. 11:01. Marked weakness in legs; animal no longer able to hop about. 11:10. Animal lying on side, not attempting to get up. 11:16. Respirations are shallow, 18 to the quarter, slower than previously. 11:19. Makes jumping motions while lying on side. Heart no longer felt. Respirations shallow. 11:21. Active peristalsis. 11:22. Several gasping respirations. Heart cannot be felt. Pupils dilated. Twitching of muscles noted. 11:23. Animal dead.

Autopsy.—Mesenteric veins markedly dilated. No hemorrhages in wall of intestine or stomach. Stomach firmly contracted. Blood removed from mesenteric vein begins to clot in half an hour; not completely clotted in forty-five minutes. Lungs not especially voluminous; show small areas of emphysema.

Heart: On opening the chest, the left ventricle is seen to be making slow ineffectual beats, the auricles are beating less frequently than the ventricles, then in dissociation. There are no pulsations in the right ventricle unless it be at the tip. The right ventricle is much distended, the muscle opaque. On opening the right ventricle, the wall contracts and becomes crinkled. On scraping, the muscle seems distinctly tougher than usual.

Other animals showed features differing slightly in details from those described. Some of them showed paralysis, beginning in the hind legs, the animals dragging themselves about. In general, muscular weakness was an early condition and death seemed to be due to cardiac, rather than to respiratory involvement. The animals showed in general the features described by Auer as occurring in acute anaphylactic shock in rabbits. A typical animal, seen by Auer, was said by him to behave and to show autopsy findings like those seen in his rabbits.

The animals dying after several hours showed at autopsy changes resembling more those seen in guinea pigs dying late. Focal hemorrhages were an almost constant finding. These were most marked, as in the guinea pigs, in the cecum and adjacent portions of the intestine, in the stomach, and in the lungs. Frequently there was a bloody exudate into the peritoneum and the bladder was filled with bloody urine.

In this series of rabbits, as in the guinea pigs, extracts of various concentration were employed and were prepared by leaving the mixtures at 37° C. or on ice for short periods of time. Typical acute death was obtained with extracts that had been kept at 37° C. for only ten minutes and also from extracts that had been prepared in the cold and kept on ice no longer than thirty minutes. It was finally found that by the injection of fifteen to twenty cubic centimeters of the extract prepared as described on page 660, acute death could almost always be produced. The tolerance of rabbits for sodium cholate was as great as four cubic centimeters of the solution in sixteen cubic centimeters of salt solution without any symptoms whatever. This is a very much larger amount than was injected in any of the experiments, and shows that the effects cannot be due to that substance.

That the result described bears a quantitative relationship to the amount of bacteria injected is shown by the following experiment:

Five rabbits were injected with various quantities of an extract prepared as previously described. One hundred c.c. of extract were prepared from the bacteria in 1 liter of culture medium. One and a half c.c. of sodium cholate solution were used in causing solution which occurred in fifteen minutes at 37° C.

Rabbit No.	Amount of extract injected.	Symptoms.	Result.
1	22 c.c.	Marked	Died, 3 min.
2	20 c.c.	Slight	Died, 4 hrs.
3	15 c.c.	Slight	Died, 11 hrs.
4	10 c.c.	Slight	Died, 12 hrs.
5	5 c.c.	None	Recovered.

Further studies dealing with the properties of the bile extract will be reported later. It may be stated, however, that tests of the effect of heating the extract gave the following results:

Guinea pig No.	Extract heated.	Result of injection. Symptoms.	Final results.
1	60–65° C. for 1 hr.	None	Recovery.
2	55° C. for ½ hr.	None	Recovery.
3	55° C. for ½ hr.	Very slight	Recovery.
4	55° C. for ¼ hr.	Very slight	Recovery.
5	45° C. for 1 hr.	Marked	Died, 30 min.

Controls with the extracts employed unheated showed marked symptoms and acute death.

Rosenow states that the toxic substance in salt solution extracts is destroyed by heating for one half hour at 60° C., but Dold[12] that the toxic extracts are quite resistant to heat; they are weakened by two hours heating to 56° to 58° C., but are not destroyed. The results of our tests of a number of bile extracts seem to show conclusively that they are thermolabile. Possibly the reason that the bodies of bacteria killed by heat are not toxic is because the thermolabile toxic substance is destroyed.

The study of the extract of pneumococcus in bile solution has shown that its toxicity for rabbits and guinea pigs is greater and more constant than is the extract in salt solution. Further work will be required to show its relation to the poison responsible for the symptoms of pneumococcus infection in animals and of lobar pneumonia in man. The fact that poisons having a similar action are found in the peritoneal exudate of infected animals suggests that these poisons may play a part in pneumococcus infection.

[12] Dold, H., Das Bakterien-Anaphylatoxin, Jena, 1912.

These observations also have a bearing on the present theories of anaphylaxis. The fact that the effects produced by this poison, both in guinea pigs and rabbits, are similar to those produced by the second injection of protein, suggests that the poisons concerned are of a similar nature, though this is not necessarily the case. In the present instance there is evidence that the poison does not arise as a result of protein digestion, since it may be set free within half an hour at a temperature of about 4° C. Proteolytic cleavage, due to ferments with which we are familiar, does not occur under these conditions. It is more likely that the toxic substances in the bile extract exist preformed in the bodies of the bacteria and are set free on their dissolution.

CONCLUSIONS.

1. The filtered blood serum of rabbits infected with pneumococci is not toxic.

2. Extracts of pneumococci prepared by keeping emulsions of the bacteria in salt solution at 37° C. for varying periods of time may be toxic, and when injected intravenously into guinea pigs, may produce a train of symptoms followed by acute death resembling that seen in acute anaphylaxis. Such extracts, however, are not uniformly toxic and it has been impossible to discover the exact conditions under which such extracts become toxic.

3. When the centrifugalized peritoneal washings of guinea pigs infected with pneumococci are injected into the circulation of normal guinea pigs, these animals very frequently exhibit symptoms like those seen in acute anaphylaxis, and a considerable proportion of the animals die acutely.

4. When pneumococci are dissolved in dilute solutions of bile salts and the solution resulting is injected intravenously into rabbits and guinea pigs, these animals show with great constancy the same symptoms that are seen in acute anaphylaxis. The solution of pneumococci in bile may occur in ten minutes at 37° C. or in half an hour on ice. This is considered evidence that the toxicity of the solution does not result from digestion of the bacterial protein, but is due to substances preformed in the bacterial cells and set free on their solution. The toxicity of the solution is diminished or destroyed by heating to 55° C. or over