THE PRODUCTION OF ANTIPNEUMOCOCCIC SERUM.

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(Received for publication, July 15, 1917.)

Ever since the demonstration in 1891 by Foa and Carbone,[1] Emmerich and Fowitzky,[2] and G. and F. Klemperer[3] of the immunizing properties of the serum of animals rendered immune to pneumococcus, this serum, modified in various ways, has been employed to a greater or less extent in the treatment of pneumonia in man.

Besides the employment of sera prepared in various ways in small series of cases, there has been an extensive employment of the sera prepared according to the directions of Pane,[4] Washbourn,[5] and more lately Römer[6] and Neufeld and Händel.[7, 8] In this country at least six commercial houses which sell biologic products have for some years been producing immune serum for the treatment of pneumonia and pneumococcus infections. It is impossible to know how extensively these sera have been employed, certainly to a sufficient extent to render their production profitable.

In spite of all that has been written concerning the theoretical principles involved in the preparation of antipneumococcic serum, and in spite of all the reports of its therapeutic application which have appeared, it is very difficult to learn from the literature on the subject exactly how these sera have been prepared or standardized.

[1] Foa, P., and Carbone, T., *Gazz. med. Torino*, 1891, xlii, 1.

[2] Emmerich, R., and Fowitzky, A., *Münch. med. Woch.*, 1891, xxxviii, 554.

[3] Klemperer, G., and Klemperer, F., *Berl. klin. Woch.*, 1891, xxviii, 833, 869.

[4] Pane, N., *Centr. Bakteriol., 1te Abt.*, 1897, xxi, 664.

[5] Washbourn, J. W., *Brit. Med. J.*, 1897, i, 510.

[6] Römer, P., *Arch. Ophth.*, 1902, liv, 99; Experimentelle und klinische Grundlagen für die Serumtherapie der Pneumokokkeninfektion der menschlichen Cornea (Ulcus serpens), Wiesbaden, 1909.

[7] Neufeld, F., and Händel, *Z. Immunitätsforsch., Orig.*, 1909, iii, 159.

[8] Neufeld and Händel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 166, 1293.

Without this knowledge we can have no accurate starting-point from which to proceed toward improvements in methods of production.

In view of these facts and also since an antipneumococcic serum is now being prepared by The Rockefeller Institute for Medical Research and used in the Hospital of this institution, and since serum made in a similar manner is now being prepared by a number of commercial houses and by several public health laboratories for wider distribution, it has seemed important that an accurate description of the methods employed by us in its production be published, together with a brief discussion of the theoretical basis for the methods employed.

Theoretical Considerations Concerning the Production of Antipneumococcic Serum.

In spite of a general belief to the contrary, all kinds of animals, even the most susceptible, may be rendered actively immune to pneumococcus infection, by the previous injection of non-lethal doses of living pneumococci or even by the injection of the dead bodies. Indeed, the substances of the bacteria which give rise to the immune reaction are very resistant to various chemical and physical agents, and a review of the literature shows that it is possible to produce active immunity with a great variety of antigens prepared from the bacteria. The degree of immunity, however, differs, depending upon the procedures employed.

The serum of the actively immunized animals, in many cases, possesses protective and curative power, the degree of this power depending somewhat on the height of the active immunity, but not invariably or regularly so. It may be stated, however, that animals whose serum is protective and curative are always actively immune. On the other hand, an animal may itself be fairly highly immune without the serum containing any immune bodies that we can demonstrate, and without its having any demonstrable protective action. For instance, we have immunized rabbits so that they have successfully withstood 0.1 cc. of a living culture, of which 0.000001 cc. killed the control, without the blood showing a trace of immune bodies or any protective power. Quite frequently animals whose serum shows only moderate grades of immunizing value may be actively resistant

to enormous doses. The fact that active immunity and serum-immunizing power do not run exactly parallel does not necessarily indicate that active immunity in pneumococcus infection differs fundamentally from passive immunity, but suggests this possibility. At any rate, it suggests that in the reaction of resistance to, or recovery from, infection there may be other factors concerned than the humoral ones. Nevertheless, with present knowledge of serum therapy our effort must be confined to the production of a serum having a high content in demonstrable antibodies and a high protective value. The chief kinds of antibodies which can be demonstrated in this immune serum are agglutinins and opsonins or bacteriotropins, and the kind of protective power meant is that which is exhibited when a very small amount of the serum is injected into a susceptible animal such as the mouse, simultaneously with, or within a few hours following, the injection of a large dose of virulent culture, which alone would cause rapid death of the animal. These are the properties of the serum which are at present tested to indicate its therapeutic strength.

It would lead us far from our present purpose, were we to discuss here at length the mode of action of the serum, for the discussion would necessarily involve a consideration of the mechanism of resistance and recovery. It is not believed that the action of the immune serum is entirely dependent either on its power of causing agglutination (Bull[9]) or on its bacteriotropic power (Neufeld and Rimpau[10] and Neufeld and Händel[7, 8]), though these properties may play important parts. They, however, are susceptible of quantitative estimation. The protection of small animals undoubtedly reproduces more accurately the part which the serum plays in recovery from natural infection, as seen in the human patient, but even here the conditions are not identical.

At the present time, however, the effectiveness of the serum seems to be parallel to its content in the antibodies we have mentioned, and especially to its protective power tested as we have described. We believe the production of antipneumococcic serum that may be accurately standardized is of fundamental importance. Whether all theoretical qualifications are fulfilled is not so essential.

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

[10] Neufeld, F., and Rimpau, W., *Z. Hyg. u. Infectionskrankh.*, 1905, li, 283.

Specificity.

The primary requisite for the serum is that it is specific. Everything stated above concerning both active and passive immunity is true only if the bacterium acted upon, either in antibody tests *in vitro*, or in protection tests *in vivo*, is identical with the organism used in producing the serum. This does not merely mean that the bacteria shall belong to the same species, for it is now generally known that different races of the same species of bacteria may show differences in their antigenic properties, even without differences in their cultural characteristics. These differences have heretofore, however, been considered differences merely in degree and mainly in lesser degree. To overcome the difficulties which this fact puts in the way of the production of immune serum for practical purposes, recourse has been had to the method of immunizing an animal with many different strains, producing a polyvalent serum. This method, for instance, has been made use of to a considerable extent in the production of antimeningococcic serum. Although certain races of meningococci differ from each other quite markedly in their immunological properties, yet they all have common characteristics which render a sharp differentiation difficult, if not impossible. The conditions as regards meningococci at present seem to be somewhat as follows. If we employ the Ehrlich nomenclature, each strain of meningococcus is endowed with a large number of kinds of receptors which we may designate by the letters of the alphabet. In one large group of strains of meningococcus *a* receptors predominate; in another group *b* receptors predominate, etc. In smaller groups other receptors, such as *k*, *l*, *m*, or *n* receptors, are most numerous. However, even in the first two groups, the *a* and *b* receptors are not exclusively present or even overwhelmingly predominating. Indeed each of the different races possesses practically all the different kinds of receptors but in greatly varying degrees.

Under these conditions, in order to produce a serum which will be active against all the different races it is necessary to choose and employ for immunization a large number of races in order that the entire receptor "spectrum" shall be covered as uniformly as possible. It seems that it is possible to do this fairly well as far as meningococcus is concerned.

The conditions as concerns pneumococcus are similar, though not identical. From agglutination experiments Kindborg[11] decided that all strains of pneumococci were immunologically distinct. Neufeld first brought evidence of group relationships and this fact has been elaborated and extended by the work in The Rockefeller Institute Hospital and elsewhere. Dochez and Gillespie[12] have demonstrated that the pneumococci obtained from cases of pneumonia occurring in this country belong in four large groups. The immunological characteristics of the organisms of three of these groups are very specific. The conditions therefore among pneumococcus are different from those obtaining among meningococcus. It is difficult to demonstrate by immunological methods that pneumococcus of Type I has any immunological characteristics (or receptors in the Ehrlich nomenclature) which are common to all pneumococci. Complement fixation tests, however, seem to show that the pneumococci of the different types do possess some common characters, at least these tests are not absolutely specific as regards the different types (Hanes[13]) and the antihemolytic reactions of specific sera against the hemolytic toxins derived from different types of pneumococci (Cole[14]) are not absolutely specific. So too the extremely active agglutinating immune sera produced by the injection of pneumococci of specific types may have slight degrees of effectiveness against certain strains of other types (Blake[15]). But in general the different types of pneumococci have a high degree of specificity as shown by protective action and by agglutination. Active immunity to the different types also seems very specific. This type specificity, which was only lately recognized, is of fundamental and primary importance in considering the practical application of immunity to therapy in this disease. Since nothing was known of this group specificity before the observations of Neufeld were made, it is very difficult to draw any conclusions from the observations or from the results of their practical application which were made in this field before that time. Where identical strains were

[11] Kindborg, A., *Z. Hyg. u. Infectionskrankh.*, 1905, li, 197.
[12] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.
[13] Hanes, F. M., *J. Exp. Med.*, 1914, xix, 38.
[14] Cole, R., *J. Exp. Med.*, 1914, xx, 346.
[15] Blake, F. G., *J. Exp. Med.*, 1917, xxvi, 67.

used throughout experiments, the conclusions of course were valid, but in the absence of knowledge of the importance of using only these strains, it is probable that in many cases this procedure was not strictly adhered to.

The work done in the Hospital of The Rockefeller Institute, both experimentally and clinically, indicates that immune serum against Type I infection is more effective than that against any other of the specific types. Indeed the results obtained both here and elsewhere indicate that this serum has great therapeutic value when it is employed in the treatment of cases due to the same type of pneumococcus. On the other hand, the observations which have so far been made with sera of the other types do not show that these produce distinct therapeutic effects. It is possible, of course, that improvements in the method of production or mode of application of these other types of serum may lead to more satisfactory results. For the present, however, we believe that the general practical application of this form of therapy should be confined to Type I cases, and this may readily be done since the type of infection in the individual case may easily be determined by the methods described elsewhere (Blake[15]). We also think it important at present that the commercial production of antipneumococcic serum for therapeutic purposes be confined to that effective against Type I infection. This paper aims to present the best method for manufacturing this serum and is based on the experience we have gained through the immunization of a very large number of rabbits, several goats, and four horses to Type I pneumococcus, four horses to Type II pneumococcus, one to Type III pneumococcus, and one to both Type I and Type II pneumococci, or ten horses in all.

Animals to Be Employed for Immunization Purposes.

For practical reasons small animals cannot be used to supply serum for therapeutic purposes, especially if the large amounts of serum which are now considered necessary are to be used.

In the earlier therapeutic tests, such as those of G. and F. Klemperer and Foa and Carbone, rabbits were used for obtaining the small amounts of serum used. Later Washbourn used ponies for immunizing purposes. Pane used a cow and an ass. Neufeld used horses in the preparation of his serum.

Römer[6] has laid stress on the importance of combining the sera prepared by immunizing a number of different animals. His reason for this is a purely theoretical one; namely, that all the different antigenic elements contained in a complex structure such as the pneumococcus may not find suitable receptors in any one animal. With present knowledge it seems that we may disregard this consideration.

We believe that for practical reasons it is best to use horses. Our experience is not sufficient to enable us to have a definite opinion as to the kind of horses best to choose for this purpose; apparently, however, draft horses are superior to the lighter grades and to the more finely bred animals. Joint injuries, indeed injuries of all kinds, undoubtedly contribute to the localization of pneumococcus and consequent focal infection. The highly strung, nervous horses are more liable to these injuries and infections. These accidents delay immunization. As regards antibody response, there has been marked individual variation, but the difference has not been associated with any obvious distinction in type of horse. In rabbits, with which our experience is much larger, there is very great individual difference. It is far more important in the production of this kind of serum than in the production of antitoxic sera that the horses should be perfectly sound and healthy, especially without old joint injuries.

Site of Injection.

Neufeld has laid stress on the value of intravenous injections. We have also employed this method since local reactions are thereby avoided. With dead cultures the local reactions are usually not severe or important; where living cultures are employed, however, they may be violent and abscess formation not infrequently occurs. It is felt, moreover, that by intravenous injections the bacteria are brought more rapidly and quickly and with less opportunity for change into contact with widely distributed cells, the reaction with which is supposed to result in the immunity response.

Choice of Antigen.

Use of Living or Dead Cultures.—As we have previously stated, the production of active immunity is attended with little difficulty and the form of antigen, so far as we know, is not of great importance,

but when we come to the question of the production of humoral immunity, especially of the highest grade, this factor may be of the greatest importance. It has generally been assumed on theoretical grounds that to produce the highest grade of humoral immunity it is necessary to use living cultures. It seems likely that all the various constituents of the bacterial cell and even some products of growth, which may be very labile, give rise to specific antibodies. Therefore to obtain the most varied and complete reaction it has been thought necessary to employ the living organisms, which may, for a short time at least, grow in the body and produce or give rise by their dissolution to certain labile metabolic substances which may stimulate antibody formation. Most of the antipneumococcic sera which have been prepared, at least of late years, have been produced by the injection first of dead, then of living organisms. The general acceptance of this method is probably due in part to what is known concerning immunity in tuberculosis. Here, as is well known, the only immunity that is effective has been produced by the injection of living organisms, by producing a mild tuberculous infection. One must be careful, however, in applying what is known concerning one type of infection directly to another, without due consideration. As knowledge of infection increases it becomes more and more evident that each disease must be considered independently. As we shall show later, it is possible that in the production of antipneumococcic serum the living organisms are not so important as they have previously been considered.

Virulence of Organisms Employed.—Pneumococci may rapidly lose their virulence for animals when grown in an artificial medium. This loss of virulence, however, may not be uniform for all kinds of experimental animals. Moreover, the virulence of pneumococci which have long been grown on artificial media may be increased by passing repeatedly through experimental animals. Here again the increase of virulence may not be uniform for all the varieties of animals. It is possible, for instance, to obtain a given strain which is very virulent for guinea pigs, with little virulence for rabbits. Even for animals as closely related in their susceptibility to pneumococcus infection as the rabbit and mouse, it is possible to have races which are highly virulent for one with little virulence for the other.

It is manifest, therefore, that we can judge only very imperfectly of the virulence of a given race for man by determining its virulence for a susceptible animal, such as the mouse or rabbit. Whether in the production of an immune serum it is important for the organisms employed to be highly virulent or not is not known. Neufeld and his associates[7, 10] have laid stress on the employment of virulent cultures, believing that the action of immune serum is to neutralize exactly those receptors of the bacterium upon which virulence depends. They state that they have proved experimentally that immunity cannot be produced with avirulent races, but give no protocols. Races virulent for mice have been employed in all our immunizing studies and we have no observations concerning immunization with avirulent races. If virulence of the organism used as antigen is important, what we have just stated concerning virulence for different species of animals becomes of great significance. It will be remembered that mice are used in all our tests of immunity. The fact that a serum is protective for mice would indicate that it had been produced by a race of pneumococci virulent for mice, but the test might give us little information relative to its protective power for man. We have therefore come to believe, on these purely theoretical grounds, that the immunization of horses should be carried on with organisms which have not been long under artificial cultivation since their isolation from the human body. These organisms are practically always virulent for mice, so that where they are employed, the test of the protective power of the serum in mice is probably a good test for protective and curative power in man. In order to have a culture which has not long been removed from the human body always ready for use, advantage may be taken of an observation made by Heim[16] and confirmed by Neufeld and Händel[7] and abundantly corroborated by us; namely, that when pneumococci are preserved in pieces of dried tissue or blood they remain viable for a very long time, and also retain their virulence undiminished. For preserving small amounts of culture the spleens of infected mice, dried and kept in a vacuum, are most satisfactory. For preserving large amounts of culture from human patients, it is well to inoculate a rabbit with blood or other infected material. After infection has reached a maximum grade,

[16] Heim, L., *Z. Hyg. u. Infectionskrankh.*, 1905, l, 123.

the rabbit is bled and the blood is spread in thin layers in Petri dishes and dried. To obtain a fresh culture at any time all that is necessary is to inoculate a little of this dried material into the peritoneal cavity of a mouse and later make a fresh culture from the heart's blood. For immunizing purposes a fresh culture obtained in this way should be prepared every 1 or 2 weeks. This precaution in obtaining cultures should be taken not only when the live bacteria are to be injected, but also when dead organisms are to be employed.

Method of Growing Organisms Used for Injection.—The use of bacteria grown in broth and separated by centrifugalization from the medium in which they are grown was first employed by Neufeld[17] who believed that the substances formed in the medium during growth are not useful but indeed harmful. We also think that it is not necessary to use the fluid in which the bacteria are cultivated. In this belief we differ from Wadsworth[18] whose observations apparently show that the serum produced with whole cultures is more effective than that produced by the injection of the bacteria alone. His experiments, however, are not entirely conclusive and in the absence of any method of accurately titrating this increased efficacy, it does not seem advisable to employ the whole culture. Moreover, the injection of the whole culture adds greatly to the difficulty of the immunizing process. As is well known, even fresh bouillon is toxic and after bacterial growth has taken place it is still more toxic. In using whole cultures, therefore, one is much restricted as to the amount that can safely be injected. When the amount of culture to be injected reaches a large size, the technical difficulties and time required in centrifugalizing the cultures become considerable. We have attempted to overcome these difficulties by growing the pneumococci on blood glucose agar in flasks and washing off the surface growth in salt solution, using the emulsion so obtained, without centrifugalizing. For certain theoretical reasons, moreover, we thought this method might be of advantage. In our experience, however, the method of growing in bouillon and centrifugalizing still proves the most satisfactory. In our earlier work the organisms were obtained by growing in broth,

[17] Neufeld, F., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 54.

[18] Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.

centrifugalizing, and washing once in salt solution, and then resuspending in salt solution. In our later work, however, we have not thought it necessary to wash, but have merely centrifugalized and then made an emulsion of the sediment in salt solution.

For obtaining a satisfactory growth in bouillon the reaction of the medium is of great importance. Pneumococci grow best in a medium the reaction of which is 0.3 to 0.5 per cent acid to phenolphthalein. If the reaction is more acid than this, a satisfactory growth may not be obtained. We have added no sugar, serum, or other enriching substance to the medium employed.

Size and Spacing of Doses.

In our first studies we employed the method which has been largely employed by others, making the injections every 7 to 8 days. This is the method generally employed in immunization and has developed from the observation that the most efficient stimulus can be applied at the time when the immunological response to the preceding dose is most active. Grades of high immunity have been thought to increase in a step-like manner, each increment being added to that previously present. It is again not certain, however, that antipneumococcus immunity obeys the same laws as other forms of immunity in which this step-like rise occurs. Certain of our observations to be mentioned later indicate that even when the injections are made at the period of greatest activity, instead of a rise in the immunity, there may be a fall, especially if the dosage is too large. After a primary immunity had been obtained by weekly injections of dead cultures, live organisms were injected, beginning with small doses, *i.e.*, the bacteria from 2 to 5 cc. of bouillon culture, and the succeeding weekly injections were gradually increased in size up to the bacteria contained in 1 or even 2 liters of culture. Neufeld speaks of injecting doses of living pneumococci as large as the bacteria obtained from 1,500 cc. of culture in the horse, and from 3,500 cc. in the ass. He injected doses of dead organisms as large as the bacteria contained in 9 liters of culture. In our experience this method of immunization is attended with many disadvantages. It has required 6 to 8 months to bring horses up to the desired grade of immunity. That these very large doses are not necessary is shown

by the following protocol of a horse in which the attempt was made to produce an effective serum by using only small doses of culture (Table I).

TABLE I.

Horse 1. Immunized to Pneumococcus Type I.

Date.	Injection.	Tests of serum.
1916		
Feb. 2	3,250 units of tetanus antitoxin subcutaneously.	
" 4	Bacteria from 25 cc. of culture, killed by heating.	
" 11	" " 50 " " " " " "	
" 18	" " 75 " " " " " "	
" 26	" " 100 " " " " " "	
Mar. 3	" living, from 2.5 cc. of culture.	
" 11	" " " 5.0 " " "	
" 19	" " " 10.0 " " "	
" 29	" " " 20.0 " " "	
Apr. 3	" " " 40.0 " " "	
" 15	" " " 80.0 " " "	
" 22		Agglutination:* complete. Protection:† 0.1 cc., D.; 0.01 cc. S.
" 22	Bacteria, living, from 120 cc. of culture.	
May 7		Agglutination:* complete. Protection:† 0.1 cc., S.

* These tests were made before we commenced the routine accurate titration of the agglutination strength.

† In the protection tests each of the mice received 0.2 cc. of serum simultaneously with a graduated dose of culture, both given intraperitoneally. The figures given indicate the amount of culture added. D. indicates died; S., survived. For brevity, only the highest dose with which recovery took place is given. In all cases the control animals receiving 0.000001 cc. of culture alone died.

It is true that the results obtained with this horse were unusually good, better than any we have since been able to obtain with a similar method. They indicate, however, that the large doses which we had been using were not necessary, that equally good results could be obtained with much smaller amounts of culture.

Modification and Improvements in the Methods of Immunization.

Over a year ago we undertook experiments to determine whether or not animals could be immunized more rapidly than had been done in the past, and also whether it might not be possible to obtain a higher grade of immunity than we had previously observed, and finally to determine for ourselves whether the use of living organisms is necessary in producing humoral immunity.

To determine the best methods of immunization a large number of rabbits was immunized in various ways and the development of immunity studied. Certain observations which had been made indicated that the process might be hastened by more frequent injections of antigen than had previously been used.

In 1900 Dean[19] showed that in the production of diphtheria antitoxic immunity the administration of the toxin at 3 day intervals gave very successful and practical results. Daily doses of antitoxin have been administered with good results when other methods have failed.[20] In 1908 Fornet and Müller[21] showed that precipitating sera could be produced very rapidly by three daily injections of antigen, bleeding on the 12th day. Bonhoff and Tsuzuki[22] confirmed these observations and Tsuzuki[23] showed that by a similar method a rapid production of typhoid-agglutinating serum could be produced. Similar observations have been made by Gay and his assistants.[24]

Flexner and Amoss[25] have employed a similar method in the production of antidysenteric serum, injecting live cultures on 3 successive days, with excellent results. In the same way the method of three daily injections has been employed by Amoss and Wollstein[26] in the production of antimeningococcic serum.

In the production of antidysenteric and antimeningococcic serum stress has been laid on regulating the size of the dose so that, following each inoculation, a febrile reaction shall be obtained. It occurred to us that the choice of three daily doses had been made more or

[19] Dean, G., *Tr. Path. Soc. London*, 1900, li, 15.

[20] Personal communication from Dr. Theobald Smith.

[21] Fornet, W., and Müller, M., *Z. biol. Techn. u. Method.*, 1908–09, i, 201.

[22] Bonhoff, H., and Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 180.

[23] Tsuzuki, M., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 194.

[24] Gay, F. P., *Ergebn. Immunitätsforsch. exp. Therap., Bakteriol. u. Hyg.*, 1914, i, 231.

[25] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

[26] Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

less arbitrarily and that possibly daily doses administered over a longer time might be still more efficacious in producing a satisfactory result, especially since this method had proved of value in the production of diphtheria antitoxin. That this method is indeed of much value is seen from the results of the experiments given below. The experiments need not be described here in detail. Certain slight variations due to external causes were made in individual instances, but most of the animals were treated about as follows.

Series 1.—These animals received weekly intravenous injections of large amounts of bacteria obtained by centrifugalization of broth cultures, which were then killed by heating for 1 hour at 56°C. The size of the doses varied from the bacteria contained in 100 cc. of broth to those contained in 500 cc.

Series 2.—These animals received intravenous injections of very small amounts of bacteria killed by heat as in the above experiment; the doses were given daily for 7 days, then an interval of 7 days was allowed to elapse, and a second series of injections was given, etc. The bacteria in the individual doses varied from those contained in 1 cc. of broth to those contained in 2 cc.

Series 3.—These animals received combined doses of immune horse serum and living cultures intravenously, beginning with 1 cc. of serum plus 0.1 to 0.5 cc. of culture. This dose was repeated every day for 3 days, then 7 days were allowed to elapse, and a second series of these combined doses was given, this time using slightly larger doses of culture. After an interval of another week, combined doses using still larger amounts of culture were given, etc.

Series 4.—These animals received varying sized doses of pneumococci killed by the addition of 0.5 per cent carbolic acid. The bacteria were centrifugalized from broth cultures, taken up in salt solution, the carbolic acid was added, and the mixture kept at 37°C. over night. As in the preceding series, the injections were given intravenously on 3 successive days, then an interval of a week was allowed to elapse, and the second series given. The number of bacteria in the individual doses varied from those contained in 2 cc. of broth to those in 50 cc. of broth. Some animals received large weekly doses subcutaneously.

Series 5.—These animals received injections of an antigen prepared as follows. The bacteria were grown in broth, centrifugalized, washed, and taken up in a very small amount of salt solution, and this emulsion was added to a large amount of acetone. The sediment which formed was centrifugalized at once, dried in a vacuum, and after 12 hours in the vacuum, taken up in salt solution and thoroughly shaken. From this somewhat viscid translucent fluid further dilutions in salt solution were made. The injections in these rabbits were made once a week, and the doses varied from an amount of antigen representing the bacteria contained in 5 cc. of broth to one representing the bacteria contained in 220 cc. of broth. The small doses were given intravenously, the larger ones subcutaneously.

In addition to the above, a small series of rabbits received injections of antigen prepared by freezing and grinding the bacteria, and another small series received injections of bacteria dissolved in bile.

In studies such as these it must be borne in mind that individual rabbits immunized in exactly the same way may show quite marked variations in their immunity response. Slight differences, therefore, in results obtained in small series of animals are not of significance.

It would lead us too far to attempt to analyze in detail the results in the various tests or to publish the protocols. One definite fact stands out from these studies. Uniformly the results following the injection of small doses of killed culture given daily over a period of 7 days followed by 7 days of rest were excellent (Series 2). This is in marked contrast to the results obtained by the injection of large doses of killed cultures given at intervals of a week (Series 1). Serum from these animals showed little or no evidence of immunity. This is strikingly shown in Table II.

TABLE II.

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
			Rabbit 1.		
1916		*gm.*			
May 27	1	1,670	Bacteria from 250 cc. of culture, killed by heating.		
	7 day interval.				
June 4	9	1,400	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 14	19	1,300	Bacteria from 250 cc. of culture, killed by heating.		
	9 day interval.				
" 24	29	1,270	Bacteria from 250 cc. of culture, killed by heating.		
	6 day interval.				
July 1	36	1,150		Undiluted, 0	0.001 cc., D.

TABLE II—*Continued.*

Date.	Day.	Weight.	Injection.	Agglutination tests.	Protection tests.
			Rabbit 2.		
1916		*gm.*			
May 28	1	1,520	Bacteria from 1 cc. of culture, killed by heating.		
" 29	2		Bacteria from 1 cc. of culture, killed by heating.		
" 30	3		Bacteria from 1 cc. of culture, killed by heating.		
" 31	4		Bacteria from 1 cc. of culture, killed by heating.		
June 1	5		Bacteria from 1 cc. of culture, killed by heating.		
" 2	6		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 10	14	1,650	Bacteria from 1 cc. of culture, killed by heating.		
" 11	15		Bacteria from 1 cc. of culture, killed by heating.		
" 12	16		Bacteria from 1 cc. of culture, killed by heating.		
" 13	17		Bacteria from 1 cc. of culture, killed by heating.		
" 14	18		Bacteria from 1 cc. of culture, killed by heating.		
" 15	19		Bacteria from 1 cc. of culture, killed by heating.		
	7 day interval.				
June 23	27	1,180	Bacteria from 1 cc. of culture, killed by heating.		
" 24	28		Bacteria from 1 cc. of culture, killed by heating.		
" 25	29		Bacteria from 1 cc. of culture, killed by heating.		
" 26	30		Bacteria from 1 cc. of culture, killed by heating.		
" 27	31		Bacteria from 1 cc. of culture, killed by heating.		
	8 day interval.				
July 6	40	1,100		1:200 + 1:400 0	0.2 cc., D. 0.1 cc., S.

As the protocols show the serum of Rabbit 1 injected weekly with large doses of culture showed no agglutinating power and only moderate protective action. On the other hand, the serum of Rabbit 2 which was treated over practically the same period of time but received very small daily doses showed high agglutinating strength, positive in a dilution of 1:200, and high protective power. These results were most surprising and striking, especially as all of the eight rabbits inoculated with the small daily doses showed an extraordinarily prompt and active response, while all of the eight rabbits inoculated with the large doses showed very little or no response in the same period of time.

The studies of other methods of immunization gave little information. The attempts to produce immunity with combined doses of serum and culture (Series 3) gave unsatisfactory results. The experiments of this series were undertaken because by this method live cultures could be injected at the very beginning of the immunization. Moreover, Theobald Smith[27] and von Behring[28] have shown the possibility of immunizing with combined doses of toxin and antitoxin. Besredka[29] and others have shown the possibility of immunizing against typhoid with sensitized cultures. Levy and Aoki[30] claim to have produced immunity to pneumococci with great rapidity (in 6 hours) by the injection of sensitized bacteria killed with carbolic acid. However, in view of what is now known concerning possible dissociation of pneumococcus antigen and antibody (Gay and Chickering[31]), it is possible that what the latter writers observed was not active immunity but slight grades of passive immunity. In any case, the grade of immunity produced was slight. Our experiments yielded no evidence in favor of the combined injection of culture and immune serum. Nevertheless, it is possible that other modifications of the method might yield better results, especially as we made no effort to balance accurately the amounts of culture and immune serum employed.

[27] Smith, Theobald, *J. Med. Research*, 1907, xvi, 359.
[28] von Behring, E., *Deutsch. med. Woch.*, 1913, xxxix, 873.
[29] Besredka, cited by Gay.[24]
[30] Levy, E., and Aoki, K., *Z. Immunitätsforsch., Orig.*, 1910, vii, 435.
[31] Gay, F. P., and Chickering, H. T., *J. Exp. Med.*, 1915, xxi, 389.

The serum of the rabbits of Series 4 inoculated with cultures killed by carbolic acid indicated no considerable grade of immunity. There is apparently no advantage to be gained in employing antigen prepared in this way. Levy and Aoki,[30] however, have reported the production of immune serum in dogs by the use of this method; but the grade of immunity, according to our standards, was slight.

Finally, the experiments in Series 5 and those in which frozen and ground bacteria and bacteria dissolved in bile were employed simply offer observations in the use of bacterial antigenic substances produced in other ways.

Neufeld[32] and Vetrano[33] have also employed bile extracts of pneumococci for immunizing purposes, only, however, in the production of active immunity. Others have employed still other artificial methods of treating the bacteria. G. and F. Klemperer[3] used glycerol extracts and Wadsworth[18] attempted immunization with cultures precipitated with alcohol and dissolved in water.

These and our own observations with artificially produced antigens show that slight grades of immunity can be produced by antigens prepared by various methods. The antigenic substance seems to be highly resistant. However, neither the observations of others nor our own indicate that these methods are especially useful in extracting the antigenic substance or in rendering it more effective. Indeed in all instances the immunity reaction resulting from the employment of antigens prepared in these ways was less intense than that following the injection of heat-killed bacteria. One fact emerges from these experiments, however, though in a less striking way than from the observations previously mentioned; namely, that small doses repeated frequently are much more effective than large doses given at longer intervals. The very large doses seem to have a definite repressing action on the development of antibodies.

These observations led us to immunize a series of four rabbits with small daily doses to determine the exact time of appearance of the immune properties in the serum. Table III is a typical protocol of one of the rabbits.

[32] Neufeld, *Z. Hyg. u. Infectionskrankh.*, 1900, xxxiv, 454.
[33] Vetrano, G., *Centr. Bakteriol., Ite Abt., Orig.*, 1909, lii, 275.

TABLE III.

Rabbit 3.

Date.	Weight.	Injection of vaccine.	Agglutination tests.	Protection tests.
1916	*gm.*	*cc.*		
May 29	1,950		0	0.0001 cc., D. 36 hrs. 0.00001 " " 36 " 0.000001 " " 36 "
" 29	1,950	1*		
" 30		1		
" 31	1,900	1		
June 1		1		
" 2		1		
" 3		1		
" 9	1,910		1:1 ++ 1:10 0	0.1 cc., D. 16 hrs. 0.01 " " 79 " 0.001 " S. 0.0001 " "
" 11		1		
" 12		1		
" 13	1,900	1		
" 14		1		
" 15	1,750	1		
" 16		1		
" 23			1:1 ++ 1:10 ++ 1:20 +	0.1 cc., S. 0.01 " " 0.001 " "
" 24		1		
" 25	1,750	1		
" 26		1		
" 27	1,900	1		
" 28		1		
" 29	1,850	1		
July 5	1,950			
" 7			1:100 ++ 1:200 + 1:400 0	0.2 cc., D. 20 hrs. 0.1 " S. 0.01 " "

* In these experiments the material for injection was prepared at the beginning of the experiment and kept on ice. 150 cc. of an 18 hour broth culture of Pneumococcus Type I was centrifugalized and the sediment washed once in salt solution. The sediment was taken up in 10 cc. of salt solution and heated ¾ hour at 56°C. Cultures were sterile. The emulsion was kept on ice and, after shaking, a small amount was removed and diluted to original volume before each injection. The injections were made intravenously.

The experiment shows that it is possible by this method of immunization to produce a high grade of immunity within 6 weeks; even within 4 weeks a considerable grade of immunity is present. The serum from each of the four rabbits showed a high agglutinating titer and a constant protective power against 0.01 to 0.1 cc. of culture. Two of the rabbits lost slightly in weight, one remained stationary, and one actually gained. They showed no other ill effects.

Experiments have also been made to show whether similar or better results could be obtained by injecting animals daily with small doses over longer periods than 7 days. Four rabbits were given small daily doses for 14 days and then bled and the serum was tested on the 5th and 10th days following the last injection. The serum of these

TABLE IV.

Horse 2. Immunized to Pneumococcus Type I.

Date.	Injection.	Agglutination test.	Protection tests.
1916			
Jan. 27	1,500 units of tetanus antitoxin subcutaneously.		
" 29	Bacteria from 50 cc. of culture, killed by heating.		
" 30	" " 50 " " " " " "		
" 31	" " 50 " " " " " "		
Feb. 1	" " 50 " " " " " "		
" 2	" " 50 " " " " " "		
" 3	" " 50 " " " " " "		
" 10	" " 50 " " " " " "		
" 11	" " 50 " " " " " "		
" 12	" " 50 " " " " " "		
" 13	" " 50 " " " " " "		
" 14	" " 50 " " " " " "		
" 15	" " 50 " " " " " "		
" 23	" " 50 " " " " " "		
" 24	" " 50 " " " " " "		
" 25	" " 50 " " " " " "		
" 26	" " 50 " " " " " "		
" 27	" " 50 " " " " " "		
" 28	" " 50 " " " " " "		
Mar. 8		1:200 +	0.1 cc., S. 0.1 " D. 0.01 " S.

animals has shown very slight agglutinating and protective power, so that this modification seems to be of no advantage.

The results of the rabbit experiments have led us to try the method of immunization described above in horses. The result obtained in one horse treated in this manner is given in Table IV.

The results in this and other horses have shown that it is possible in this way to produce a very high grade of primary immunity with a great saving in time and without danger to the animal. The dosage has been arbitrarily chosen. It is possible that with further experience and more carefully regulated dosage it may be possible to produce even higher grades of immunity in this way. It may even be possible by this method to obviate entirely the use of live cultures.

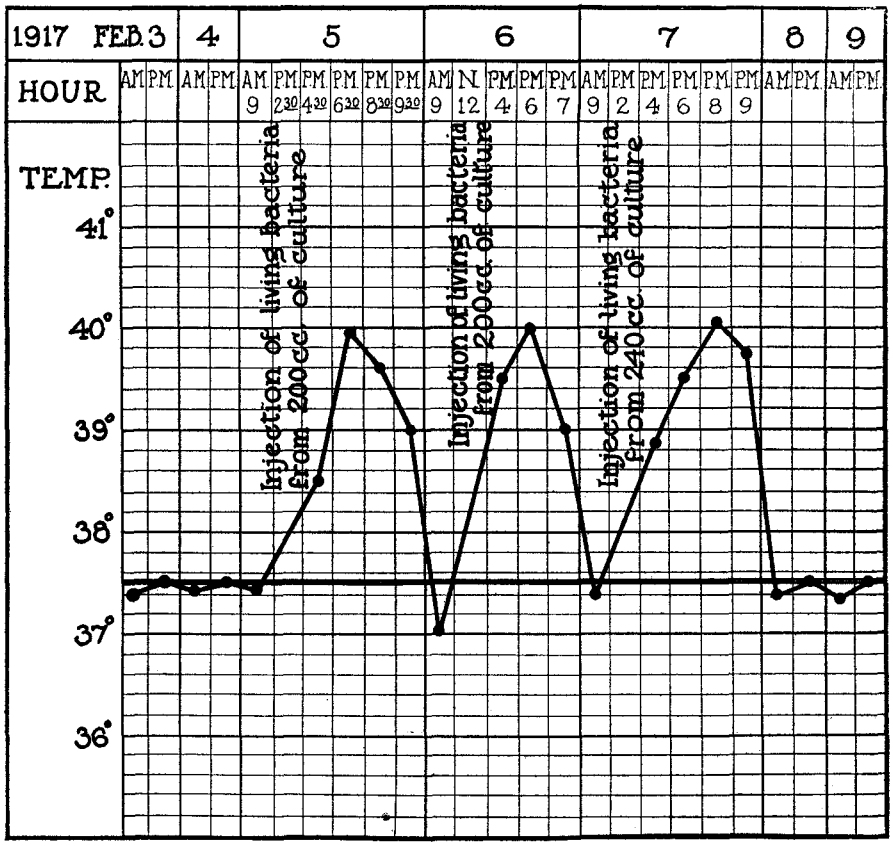
What has been attempted so far, however, has been the production of a primary immunity in the quickest possible time and with the least loss in horses.

TABLE V.

Horse 2, Undergoing Immunization Since January 29, 1916. Last Bleeding, 11 Liters, on May 15, 1917.

Date.	Injection.	Protection tests.
1917		
May 26	Bacteria from 100 cc. of culture, killed by heating.	
" 27	" " 100 " " " " " "	
" 28	" " 100 " " " " " "	
" 29	" " 100 " " " " " "	
" 30	" " 100 " " " " " "	
June 1	" " 100 " " " " " "	
" 9	" " 100 " " " " " "	
" 11	" " 100 " " " " " "	
" 14	" " 100 " " " " " "	
" 15	" " 100 " " " " " "	
" 16	" " 100 " " " " " "	
" 17	" " 100 " " " " " "	
June 25	Blood test.	0.01 cc., D.
July 6	Bacteria, living, from 100 cc. of culture.	
" 7	" " " 130 " " "	
" 8	" " " 160 " " "	
" 13	Blood test.	0.2 cc., S.

The problem now confronting us is to determine the method by which horses having an established primary immunity may be brought up to the highest possible level and kept there. In order to do this, when dead cultures have failed, it may be necessary to resort to living



TEXT-FIG. 1. Temperature curve of Horse 3 injected with living Pneumococcus Type I.

cultures. An observation on one of our horses makes this probable (Table V).

In this horse, whereas repeated small daily doses of killed culture failed to bring the serum up to full strength, three moderate sized doses of living culture caused the serum to acquire maximum power.

This result has been seen on numerous occasions and it is probable that in most horses living cultures must finally be employed. When injections of living cultures are made we now employ the method described by Flexner and Amoss,[25] administering three doses in amounts sufficient to produce a moderate febrile reaction. A typical curve is shown in Text-fig. 1. So far as pneumococcus immunity, however, is concerned, the necessity for producing febrile reactions is not established. Neufeld believed that so far as agglutinating sera are concerned the power depends not so much on the height of the immunity as on the intensity of the last reaction through which the animal had passed. Therefore he thought it advisable to inject as large amounts as possible without killing the animal. The observations we have made do not support this point of view. In our experience with horses a violent reaction is not always followed by a marked immunity response or increase in agglutinating power of the serum; indeed the opposite is frequently the case. For the present, however, in giving live cultures it is probably better to be guided in the size of the dose by the febrile reactions. We think, however, that large doses should be avoided even though the febrile reaction is slight.

Typical Method of Immunization Based on Previous Observations.

As a result of our observations and those of others we now believe that immunization should be carried out according to the method as at present employed by us, which is briefly given below.

Having obtained a sound, fairly heavy horse, it is first given a glanders test. At present the complement fixation test is used for this purpose. A specimen of 20 cc. or more of blood is obtained before any treatment is given and is kept for use in control tests. The immunization is then carried out as follows. All injections are made intravenously, employing for this purpose a Luer syringe. To avoid any accidental injury to the vein it is well to have the needle attached to the syringe with a small piece of rubber tubing. The culture used for injection, whether living or dead, should be one highly virulent for mice, 0.000001 cc. killing regularly, and it should have gone through very few passages in animals or on artificial media since removal from the human patient. The method for keeping the cultures is described above (page 545). In preparing the material for injection, both living and dead, the organisms are grown on beef peptone broth, reaction 0.3 to 0.5 per cent acid to phenolphthalein. Cultures about 12

to 15 hours old are preferable as at this time maximum growth is present, with a minimum of autolysis (Chesney[34]). The cultures should contain about 200 to 300 million bacteria per cc. The culture is centrifugalized until the supernatant fluid is clear. With the large centrifuge employed by us this requires about 20 to 30 minutes. The supernatant fluid is then poured off and the sediment is taken up in a small amount of sterile salt solution.

If the organisms are to be injected alive, the emulsion in salt solution is not made until just before injection, so that autolysis and death may not take place. If the organisms are to be injected dead, the emulsion is placed in a tube in a water bath and kept at 56°C. for ¾ hour. For the daily injections a considerable amount of the emulsion, after killing, is prepared and kept on ice. This may be employed for all the injections in the series of 6 to 7 days. We think, however, a fresh emulsion should be prepared each week. For each injection the dilution of the fluid should be such that the volume injected is about 20 cc.

The following course of injections is now carried out. Every day for 6 days an amount of the emulsion of killed pneumococci containing the bacteria from 50 cc. of the bouillon culture is injected. An interval of 7 days is allowed to elapse and then a second series of daily injections, of the same size, is made. Again an interval is allowed to elapse and on the 6th day a specimen of blood is obtained for testing. Tests are made at once for agglutinating and protective power. This requires several days.

If the serum causes agglutination in a dilution of 1:200 and is of standard protective value, 0.2 cc. regularly protecting a mouse against 0.1 cc. of a virulent culture, bleeding may be carried out at once; that is, on the 10th to 12th day following the last injection. As a matter of fact, we have never seen the titer of the serum after this amount of treatment to be so high. Consequently it has been our practice, and we advise, that 8 to 10 days after the last injection of the second series of dead bacteria, injection of live organisms be commenced. These injections are given on successive days. The first injection should consist of the bacteria contained in 20 cc. of the original culture. The temperature is taken every 2 hours for 8 to 10 hours following each of the injections of live cultures. If the temperature reaction is only moderate, not over 40.5°C., an injection of the bacteria from 40 cc. of culture is given on the following day. If the reaction from this is only moderate, the dose is again doubled on the following day and the bacteria from 80 cc. of culture are injected. As before stated, so far as pneumococci are concerned it is difficult to regulate the dosage entirely by the febrile reaction obtained. If the reaction is very severe, of course the dose is made smaller than those mentioned. On the other hand, even though the reaction is very slight, we do not advise giving more than the amounts stated. 6 days after the last injection another specimen of blood is obtained for testing. If the serum is of the standard strength, bleeding can now be done. If it is still too weak, a second

[34] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

series of injections of living bacteria is made. These injections should consist of the bacteria from 100 cc., 150 cc., and 200 cc. of broth on the 3 days respectively. Again, the size of the doses may need to be somewhat modified, on account of the severity or lack of febrile reaction. Certain horses may require still further injections of live cultures but this is exceptional. In any case, we think the injections should be made in series of three, given on successive days, with 7 day intervals between each series, and from our present standpoint we believe that the size of the injection should never be greater than the bacteria from 300 to 400 cc. of broth. Following the bleeding, it is well to allow the horse to remain quiet for 3 or 4 days. Then a series of three injections of living cultures, 50, 80, and 100 cc., is again given. After a week the serum is again tested and if of standard strength bleeding may again be done on the 10th day following the last injection.

CONCLUSIONS.

In the production of immune serum for therapeutic purposes strict attention must be paid to the immunological specificity of the bacteria used for immunization. At present the only serum of which the therapeutic value has been proven is that effective against Type I pneumococcus infection. This serum should have agglutinating power for Type I pneumococcus and should have the power of protecting mice against large amounts of virulent culture. Experiments have shown that for producing the primary immunity most rapidly several series of small doses of dead cultures should be given, the injections being made daily for 6 to 7 days, followed by a week in which no injections are made. To produce the highest type of immunity probably living organisms are required. These should be given in moderate doses daily for 3 days, with an interval of a week between each series of injections. By following accurately the methods described, horses may be made to produce rapidly a high grade of specific serum. The observations so far made indicate the importance of employing small doses of culture frequently repeated in this form of immunization.