A SPECIFIC FLOCCULATION REACTION OCCURRING BETWEEN ALCOHOLIC EXTRACTS OF PNEUMOCOCCI AND ANTIPNEUMOCOCCUS SERUM.*

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INTRODUCTION.

The study of the antigenic properties of lipoidal substances extracted from antigenic material by alcohol, ether, or other fat solvents has included investigation of the significance of lipoidal constituents of the red blood cell in the formation of lysins and agglutinins, the action of alcoholic extracts of organs in the production of heterogenetic antibodies and their reaction with Forssman's antigen, and, finally, of the specific nature of such lipoidal fractions obtained from bacterial cells. Until recently, experimental work on the bacterial cells had been limited almost entirely to the immune reaction in tuberculosis. No attempt is made to review the large amount of work carried out in this particular field by numerous authors, as this has lately been done by Wadsworth, Maltaner, and Maltaner (1). In their study of the complement fixation reaction in tuberculosis, they report a systematic investigation into the distribution of the antigenic properties in tubercle bacillus cultures, which they found to be associated with the acetone-insoluble lipoids.

While the rôle of non-specific lipoids, extracted from various tissues, in the numerous flocculation reactions devised for the serodiagnosis of syphilis, has long been recognized, the action of bacterial lipoids in a specific flocculation reaction had not been made the subject of extensive research until 1924 when Dujarric de la Rivière and Roux

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(2) described flocculation occurring between alcoholic extracts of meningococci and antimeningococcus serum.[1] They found that the combination of antimeningococcus serum with watery emulsions of alcoholic extracts of meningococci, reinforced with tincture of benzoin, would result in macroscopically visible specific flocculation, which they considered to be immunologically different from either the agglutination or the complement fixation reaction. The authors suggest the value of this reaction in the standardization of antimeningococcus serum. More recently, they (3) have also included the gonococcus in their studies. Georgesco (4) has reported the application of the same method to the serodiagnosis of cerebrospinal meningitis and typhoid fever. Lately, Klopstock (5) has obtained very significant results with alcoholic extracts prepared from pure cultures of *Treponema pallidum* in the complement fixation reaction for syphilis.

While engaged in a study of this flocculation reaction with the meningococcus, during which it was possible to confirm Dujarric de la Rivière's results with serums and extracts prepared in this laboratory,[2] it was decided to investigate whether a flocculation reaction of similar character occurring under essentially the same conditions, could be obtained with alcoholic extracts of pneumococci and antipneumococcus serum. Experimental work on this subject was prompted, partly, by the desire to ascertain whether such a flocculation reaction between bacterial lipoids and immune serum was not only species- but also type-specific. It is unnecessary to emphasize particularly in this connection that, in comparison with the pneumococcus, the types of meningococci at present appear to be less sharply differentiated. In the foreground of interest, however, stood the

[1] An alcoholic extract of meningococci prepared by grinding meningococci with silica and subsequently extracting the bacteria at 37°C. with 96 per cent alcohol was proposed by I. R. Mörch in 1922 (reported by T. Madsen in Reports on Serological Investigations, League of Nations, Health Organization, 1923, 35) as a standard antigen for testing the potency of antimeningococcus serum. This extract, diluted with salt solution, was mixed with decreasing amounts of serum and the mixture incubated for 18 hours at 37°C. A specific precipitation reaction occurred, which was found to provide a basis for the estimation of the therapeutic potency of the serum.

[2] Dr. Dujarric de la Rivière of the Pasteur Institute in Paris kindly provided us with reagents for comparative work with the meningococcus.

study of the relation of this new immune reaction to the therapeutic potency of the immune serum. Obviously, this would meet with obstacles in the case of antimeningococcus serum since no satisfactory test to measure unmistakably the therapeutic potency of this serum has yet been described, and the substantiation of its efficacy by clinical data is extremely difficult and unreliable. This problem would appear to be more accessible to study with the pneumococcus, inasmuch as the standardization of antipneumococcus serum, based on the protection test in mice, reflects with a high degree of accuracy the therapeutic potency of this serum.

EXPERIMENTAL WORK.

Antigens.—The bacterial antigens were prepared by extracting the centrifugalized, washed sediment of 1500 cc. of an 18 hour pneumococcus broth culture in 40 cc. of absolute or 95 per cent ethyl alcohol for a period of 4 weeks, at room temperature. The extracts were then filtered through paper and were ready for use. Antigens obtained by extracting the bacterial sediment for 2 hours with boiling alcohol in a reflux condenser were found to be only slightly weaker in flocculating power than those prepared by the first method. The cultures used were: Three Type I strains—two standard strains, Pneumococcus Type I No. 5 (Neufeld) and Pneumococcus Type I No. 5D (U. S. Hygienic Laboratory), and a Pneumococcus Type I strain recently isolated in this laboratory from pneumonic sputum; one Type II strain and one Type III strain, both the latter isolated at and received from the Hospital of The Rockefeller Institute. The extracts used for the major part of the work described in this paper were prepared from Pneumococcus Type I No. 5.

It seemed of interest to obtain some information on the chemical and biological nature of the substances extracted by the alcohol.

5 cc. of extract, Type I, were evaporated, and the dry residue, 6 mg., was resuspended in 2 cc. of physiological salt solution, which produced an almost clear solution. It gave no biuret reaction, but reacted very slightly with Millon's reagent. A Molisch test resulted in a very faint purple color comparable to the one obtained with a 1:500,000 dextrose solution. When the solution was overlaid in 0.3 cc. amounts on 0.3 cc. of each of the tree types of antipneumococcus serum, in no instance did the solution give a precipitin reaction (incubation up to 3 hours at 37°C.). The extracted Type I organisms, on the other hand, suspended in salt solution, were still specifically agglutinated by Type I antipneumococcus serum, although they had become insoluble in bile. This suspension was boiled for a short time, to obtain some of the soluble substances in solution, filtered, and the clear filtrate tested for a precipitin reaction with antipneumococcus serums of

Types I, II, and III. A very distinct type-specific precipitation occurred. In order to obtain information on the antigenic properties *in vivo* of the substances extracted by the alcohol and of the bacterial cells after extraction, 20 cc. of alcoholic extract were evaporated, and the dry residue was resuspended in 15 cc. of salt solution. Simultaneously, the bacterial cells after extraction, representing the treated sediment of 750 cc. of broth culture, after several washings, were suspended in 15 cc. of salt solution. One rabbit was given the total volume of the first preparation divided into four subcutaneous injections; another rabbit, the total volume of the second preparation similarly divided. Both animals were bled on the 5th day following the last injection. While the serum from the rabbit which had received the emulsion of the residue of the alcoholic extract gave neither an agglutination nor a precipitin reaction, the serum of the rabbit treated with the bacterial cells after extraction with alcohol, agglutinated faintly up to a 1:2 dilution and gave, undiluted, a distinct precipitin ring with the filtrate of a 48 hour autolyzed pneumococcus broth culture. The two rabbits were then tested for the degree of immunity developed, by the intravenous injection of 0.001 cc. of a highly virulent pneumococcus culture which had been passed through rabbits for a number of years. This dose, which killed a normal control rabbit in 48 hours, killed the rabbit which had received the emulsion of the residue of the alcoholic extract in 72 hours, while the other animal, immunized with the alcohol-treated bacteria, survived. The same animal, when injected 4 days later with 0.1 cc. of the same culture, was fully protected.

Serums.—The serums used for the tests were monovalent antipneumococcus serums obtained from horses immunized by various methods. Incidentally, a small number of immune serums from rabbits immunized with the whole pneumococcus culture for various periods of time, were tested. Finally, it has been possible to examine several samples of serum from pneumonia convalescents, obtained through the courtesy of Dr. Cole from the Hospital of The Rockefeller Institute in New York City and from Dr. Ordway of the Albany Medical College.

The Test.—In setting up the test, Dujarric de la Rivière's original method was modified in many respects. The addition of an unspecific reinforcing agent to the specific alcoholic bacterial extracts proved to be indispensable in order to make the flocculation reaction macroscopically readily visible. A 10 per cent alcoholic extract of Sumatra benzoin[3] was used for this purpose after it had been found that lecithin and cholesterol, although capable of reinforcing the intensity of the reaction considerably, did not induce as rapid a flocculation as did the benzoin.

[3] It would seem advisable to use no benzoin tincture which is older than 2 months, since evidence of deterioration was noted after that time. The bacterial antigens, on the other hand, appear to be stable for a longer period.

The technique of the test as finally adopted is briefly as follows:

To one part of benzoin tincture are added twelve parts of alcoholic bacterial antigen. This initial mixture is suspended in 0.85 per cent salt solution by adding quickly 25 cc. of the salt solution to 0.4 cc. of the initial mixture. It is important to add the diluent very quickly. Experiments in which buffered salt solutions of different pH values were used in preparing this suspension, indicated a neutral medium as the most desirable for the reaction. The colloidal suspension thus obtained is slightly opaque and just on the verge of spontaneous flocculation; if left standing at room temperature or shaken vigorously, it will flocculate by itself. In order to insure conditions of maximum sensitivity, it is necessary to determine for each new antigen and new benzoin tincture the optimum balance between bacterial antigen, benzoin, and salt solution. A constant volume of 2.5 cc. of this suspension is mixed with varying amounts of serum, starting with 0.5 cc. and ending with 0.05 cc. It is not advisable to test smaller amounts than 0.05 cc. because it was found that less than 0.05 cc. of normal serum is insufficient to stabilize the suspension; the comparable range of specific flocculation is thus limited to the amounts indicated. The tubes are incubated, half immersed in a water bath at 40°C. since this temperature was found to provide conditions of the greatest sensitivity, maintaining at the same time the highest degree of specificity; it was observed that normal horse serum flocculated slightly above 46°C. Readings are made after ½ hour, 1 hour, and 2 hour intervals to determine the degree of flocculation. Occasionally, another reading is made on the following day.

Results.—It was found that a number of different antipneumococcus, Type I, Type II, and Type III serums, when tested in the manner described, gave a flocculation reaction of varying intensity with their homologous alcoholic bacterial antigens. While a great many of the fresh Type I serums gave a marked flocculation with Type I antigens, two older Type II serums and one older Type III serum, the only serums of these types available, reacted only faintly with their respective antigens. The species specificity of this reaction was controlled (1) by testing several normal horse serums as well as other immune serums, such as, antimeningococcus serum and antidysentery serum, with the pneumococcus antigen, (2) by testing alcoholic extracts of other microorganisms prepared, in a similar way, with antipneumococcus serum. In no case was flocculation observed under such conditions within the limits of the experiment.

In order to determine whether this reaction exhibited also type specificity, pneumococcus antigens, Types I, II, and III, were tested with the heterologous type serums. Some slight cross-reactions oc-

curred between Type II and Type III antigens and Type I serum as recorded in Table I, but no overlapping was noted with Type I antigen.

It was soon found that a number of Type I serums older than approximately 1 year, flocculated slightly, if at all, in spite of the fact that they still agglutinated and protected very well. It must remain undecided at present whether serums lose their flocculating power by aging, because no serum giving flocculation has yet been observed for longer than about 4 months. For the same reason, it is impossible to state whether the weak reactions obtained with two Type II serums and one Type III serum with their homologous antigens are attributable to the age of the serums, which were between 1 and $2\frac{1}{2}$ years old, or whether flocculation runs parallel to the protective action of antipneumococcus serum in general, having no relation to the agglutinative titer. As is usually the case, these Type II and Type III serums had very little, if any, protective action, while their agglutination titer was approximately the same as that of the Type I serums.

The results of these tests are tabulated in Table I.

A number of serums from rabbits immunized with pneumococcus, Type I, were tested for flocculation. While the reactions with these rabbit serums, which had a comparatively high agglutination titer, were very weak, the tests were too few to be of much significance. There is some reason to believe that, after prolonged immunization, the serum may acquire definite flocculating power.

The results obtained with the pneumonia convalescent serums are still too scanty to warrant a detailed report, although definite flocculation occurred in several instances with serum taken as early as the 2nd day of the disease. Occasionally, however, a very slight flocculation with the largest amount of normal human serum tested, 0.5 cc., was noted. In this connection, it should be recalled that Wadsworth (6), in his early work, obtained a precipitin reaction with high dilutions of convalescent pneumonia serums by using extracts of pneumococci. The centrifugalized fresh pneumococcus cells had first been shaken with hypertonic salt solution to increase the plasmoptysis, the extract then being made isotonic by the addition of water. He also observed a reaction with serums from apparently

healthy persons, in a dilution of 1:10, after 16 hours. It is hoped to improve the technique in future work so that significant results may be secured.

Further study was directed to a closer investigation of the nature of this flocculation reaction. It was found that supernatant serum which had flocculated, would not flocculate again, while the agglutination titer and the protective action of such a serum had remained practically unaffected; the solution of the flakes in salt solution up to the original volume released no protective action. Next, the effect of absorption was studied. It appeared that Pneumococcus Type I serum which, after absorption for 6 hours at room temperature with Type I organisms, had lost completely its agglutinative action, showed no flocculation whatsoever for the first 2 hours of incubation. Left overnight at room temperature, there was, however, considerable flocculation, although much less than occurred in the non-absorbed serum even after 2 hours' incubation. Later, the effect of heating for $\frac{1}{2}$ hour and 1 hour at 56°C. was studied in two different Type I serums. The results obtained did not agree. While heating for $\frac{1}{2}$ hour impaired the flocculating power of one serum considerably and heating for 1 hour completely destroyed it, the other serum, after being heated for $\frac{1}{2}$ hour, flocculated almost as well as the non-heated serum and even heating for 1 hour did not destroy its flocculating power. The agglutinative action of these two serums was similarly affected by heating as was the flocculating power. The addition of complement, in the form of fresh normal horse or guinea pig serum, did not reactivate the heated serum nor did the addition of complement to old serums improve their flocculating power.

Finally, the relation of this flocculation test to the therapeutic potency of Type I antipneumococcus serum was studied. Over forty serums obtained from ten different horses at various stages during immunization were tested for flocculation, and the results of these tests were compared with the protective titer ascertained by the routine method of standardization in mice. Although it had already been observed that the serums in repeated tests exhibited approximately the same flocculating power on different days, a serum which flocculated well was chosen as a standard and run along simultaneously with normal horse serum with each test, as a control. The results are tabulated in Table II.

TABLE I.

*Experiments on Type and Species Specificity of Flocculation Reactions with Alcoholic Extracts of Pneumococci.**

Pneumococcus Type I antigen										Pneumococcus Type II antigen									
Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for			Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for		
		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.			½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.
Pnc. Type I Serum 312	1.7.26	2+	3+	4+	2+	2+	3+	±	1+	1+	Type I Serum 312	1.7.26	−	±	1+	−	±	±	−	−	−
" " I " 313	1.26.26	2+	3+	4+	2+	3+	4+	2+	3+	3+	" I " 313	1.26.26	−	±	1+	−	±	±	−	−	−
" " II " 93	10.22.23	−	−	−	−	−	−	−	−	−	" II " 93	10.22.23	−	±	1+	−	±	±	−	−	−
" " II " 85	7.16.23	−	−	−	−	−	−	−	−	−	" II " 85	7.16.23	−	±	±	−	−	−	−	−	−
" " III " 62	1.4.25	−	−	−	−	−	−	−	−	−	" III " 62	1.4.25	−	−	−	−	−	−	−	−	−
Normal Horse Serum (H. 144)	4.24.25	−	−	−	−	−	−	−	−	−	Normal Horse Serum (H.144)	4.24.25	−	−	−	−	−	−	−	−	−
" " " (H. 140)	2.5.24	−	−	−	−	−	−	−	−	−											
" " " (H. 140)	2.2.26	−	−	−	−	−	−	−	−	−											
Antimeningococcus Serum 145	3.3.25	−	−	−	−	−	−	−	−	−											
Antidysentery Serum 75	2.7.25	−	−	−	−	−	−	−	−	−											

Pneumococcus Type III antigen										Antigens from other microorganisms tested with Type I Antipneumococcus Serum 312									
Serums	Date bled	Mar. 9 0.5 cc. of serum incubated for			Mar. 9 0.3 cc. of serum incubated for			Mar. 9 0.1 cc. of serum incubated for			Antigens	Mar. 10 0.5 cc. of serum incubated for			Mar. 10 0.3 cc. of serum incubated for			Mar. 10 0.1 cc. of serum incubated for		
		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.		½ hr.	1 hr.	2 hrs.	½ hr.	1 hr.	2 hrs.	½ hr	1 hr.	2 hrs.
Type I Serum 312	1.7.26	−	−	±	−	−	−	−	−	−	*Streptococcus viridans*	−	−	−	−	−	−	−	−	−
" I " 313	1.26.26	−	−	1+	−	−	−	−	−	−										
											Micrococcus catarrhalis	−	−	−	−	−	−	−	−	−
" II " 93	10.22.23	−	−	−	−	−	−	−	−	−										
" II " 85	7.16.23	−	−	−	−	−	−	−	−	−	Meningococcus 10-B	−	−	−	−	−	−	−	−	−
											" W 30-B	−	−	−	−	−	−	−	−	−
" III " 62	1.4.25	±	1+	1+	±	1+	1+	−	−	−	" 46-B	−	−	−	−	−	−	−	−	−
											" 79-B	−	−	−	−	−	−	−	−	−
Normal Horse Serum (H. 144)	4.24.25	−	−	−	−	−	−	−	−	−										
											Alcohol	−	−	−	−	−	−	−	−	−

* The intensity of flocculation is designated as follows:

− = diffuse opacity.
Tr. = trace (faintest granulation).
± = slight granulation.
1+ = small flakes.
2+ = medium sized flakes.
3+ = large flakes.
4+ = heavy flocculent precipitate at the bottom leaving supernatant fluid clear.

TABLE II.

Comparison between Flocculation Reaction, Agglutination Titer, and Protective Action of Different Type I Antipneumococcus Serums.

Horse and bleeding Nos.	Serum No.	Immunization	Date bled	Dates of flocculation tests 1926	Flocculation after 1 hr.'s incubation at 40°C.			Date of agglutination test	Agglutination after 2 hrs.' incubation at 37°C.				Date of protection test	Protective action	
					0.5 cc.	0.3 cc.	0.1 cc.		1:10	1:20	1:40	1:80		Standard serum	Test serum
														0.2 cc. protected	
														Culture	
														cc.	*cc.*
143–10	271	11 mos.	7.24.24	2.5 2.9	Tr.	Tr.	–	9.11.24	3+	3+	–	–	9.17.24	0.1	0.2
–11	273	11 "	7.29.24	2.5 2.9	2+	2+	±						9.17.24	0.1	0.2
–12	279	1 yr. 3 mos.	1.3.25	2.5 2.9 2.11	Tr.	Tr.	Tr.						1.7.25	0.1	0.3
152–13	270	1 yr.	7.16.24	2.5 2.9	–	–	–	9.11.24	2+	–	–	–	7.31.24	0.1	0.1
–14	272	1 "	7.29.24	2.5 2.9 2.11	±	Tr.	Tr.						9.17.24	0.1	0.2
190–3	289	4 mos.	4.22.25	1.29 2.5	±	–	–	2.1.26	4+	2+	–	–	5.13.25	0.1	0.1*
189–4	290	5 "	5.6.25	1.29 2.5	1+	Tr.	Tr.	7.17.25	3+	3+	1+	1+	5.28.25	0.2	0.1
193–3	303	105 days	11.7.25	2.21 2.24	±	–	–	3.18.26	3+	2+	1+	±	11.11.25	0.1	0.1*
–4	309	136 "	12.8.25	2.21 2.24	3+	2+	1+	2.1.26	3+	3+	2+	–	12.9.25	0.1	0.2
–5	310	142 "	12.14.25	2.21 2.24	3+	2+	2+	3.18.26	3+	3+	3+	2+	12.16.25	0.1	0.1
–6	311	164 "	1.5.26	2.21 2.24	3+	3+	2+	3.18.26	3+	3+	3+	1+	1.6.26	0.1	0.1
–7	313	185 "	1.26.26	2.21 2.24	3+	3+	3+	3.18.26	2+	3+	3+	–	1.27.26	0.1	0.1
–8	316	207 "	2.17.26	2.21 2.24	2+	2+	1+	3.18.26	3+	3+	2+	–	3.4.26	0.1	0.1
–9	318	221 "	3.2.26	3.5 3.7	2+	1+	1+	3.18.26	3+	3+	2+	–	3.16.26	0.2	0.2
161–3	302	105 "	11.7.25	2.11 2.24	2+	1+	–	3.18.26	3+	2+	±	–	11.11.25	0.1	0.1*
–4	307	135 "	12.7.25	2.11 2.24	1+	Tr.	–	3.18.26	3+	3+	1+	–	12.9.25	0.1	0.1**

−5	312	166 days	1.7.26	2.11	2.24	3+	2+	1+	3.18.26	3+	2+	2+	−	1.13.26	0.1	0.1
−6	314	188 "	1.29.26	2.11	2.24	3+	3+	1+	3.18.26	3+	3+	1+	−	2.10.26	0.1	0.1
−7	317	215 "	2.20.26	3.5	3.17	1+	±	±	3.18.26	3+	3+	−	−	3.4.26	0.1	0.2
−8	322	231 "	3.13.26	3.17	3.19	±	±	−	3.18.26	3+	3+	−	−	3.16.26	0.2	0.1
−9	323	244 "	3.27.26	4.9		−	−	−	4.28.26	3+	2+	−	−	3.31.26	0.1	0.1*
−10	327	263 "	4.16.26	4.20		±	±	−	4.28.26	4+	−	−	−	4.21.26	0.1	0.1
201–1	306	111 "	11.13.25	2.5	2.9	1+	±	−	3.18.26	3+	1+	±	−	11.18.25	0.2	0.1
−2	308	136 "	12.8.25	2.5	2.9	2+	1+	±	2.1.26	3+	−	−	−	12.9.25	0.1	0.1
207	Norm. bl.	0	12.19.25	2.23	2.26	−	−	−								
	1st trial bl.	38 "	1.26.26	2.23	2.26	−	−	−						2.3.26	0.3	0.1**
	2nd " "	56 "	2.13.26	2.23	2.26	−	−	−	3.18.26	3+	1+	−	−	2.17.26	0.1	0.1**
−1	320	82 "	3.11.26	3.15	3.17	1+	±	−	3.18.26	3+	3+	±	−	3.16.26	0.2	0.1
−2	325	103 "	4.1.26	4.9		−	−	−	4.28.26	3+	3+	1+	−	4.14.26	0.1	0.2
−3	328	124 "	4.22.26	4.22		1+	1+	±	4.28.26	4+	2+	−	−	4.26.26	0.1	0.2
−4	331	147 "	5.13.26	6.1		1+	1+	1+	5.19.26	3+	3+	−	−	5.19.26	0.2	0.2
150	Norm. bl.	0	11.27.25	2.23	2.26	−	−	−								
	3rd trial bl.	64 "	1.30.26	2.23	2.26	−	−	−	4.28.26	3+	1+	−	−	2.3.26	0.2	0.1
	4th " "	73 "	2.8.26	2.23	2.26	−	−	−	3.18.26	3+	3+	−	−	2.10.26	0.2	0.1
−1	315	82 "	2.17.26	2.23	2.26	Tr.	−	−	3.18.26	3+	2+	1+	−	2.24.26	0.1	0.1
−2	319	96 "	3.3.26	3.15	3.17	±	−	−	3.18.26	3+	2+	±	−	3.10.26	0.1	0.1
−3	324	124 "	3.31.26	4.9		2+	2+	1+	4.28.26	3+	3+	±	−	11.15.26	0.1	0.1†
151	Norm. bl.	0	12.19.26	3.5	3.15	−	−	−								
	1st trial bl.	38 "	1.26.26	3.5	3.15	−	−	−						1.27.26	0.1	0.1**
	2nd " "	56 "	2.13.26	3.5	3.15	−	−	−	3.18.26	3+	−	−	−	2.17.26	0.1	0.1**
−1	321	82 "	3.11.26	3.15	3.17	1+	±	−	3.18.26	3+	2+	1+	−	3.16.26	0.2	0.2
−2	326	103 "	4.1.26	4.9		−	−	−	4.28.26	4+	1+	−	−	4.21.26	0.1	0.1
−3	329	124 "	4.22.26	4.29		−	−	−	4.28.26	4+	2+	−	−	4.26.26	0.1	0.1
−4	330	147 "	5.13.26	6.1		±	±	±	5.19.26	4+	2+	±	−	5.19.26	0.2	0.2

* Protected partially. ** Did not protect.

† Earlier tests of the serum, while irregular, suggested its value to be approximately equal to that of the standard serum.

In studying Table II, Horses 193 and 161 attract particular attention, inasmuch as it had been possible to follow a number of bleedings taken between the 3rd and the 8th or 9th month of immunization, and to compare the results of the protection test in mice and the agglutination titer with the flocculating power as measured by the test described. A relationship between the flocculating power of the serum from these horses and the protective action is suggested. In the case of Horse 161, a marked decrease in the flocculating power of the serum occurred before the end of immunization. This horse was in very poor physical condition when bled out. It is also of special interest to note that Horse 193 developed endocarditis toward the end of immunization, which condition apparently did not affect the protection titer of the serum, but there was a slight drop in the flocculating power. Horses undergoing active immunization by the intravenous inoculation of living virulent pneumococci frequently develop lesions in the heart valves, as first described by Wadsworth (7), in 1919, and yet their serums possess high protective action in mice against virulent pneumococci. In Horse 150, the flocculating power developed very much more slowly than the protective action and, in Horses 151 and 207, even at the end of the 5th month of immunization, there was only slight flocculation, although three previous bleedings of Horse 151 and two previous bleedings of Horse 207 had given a protection value at least equal to that of the standard serum. The agglutinative activity, as evidenced especially by Horses 207, 150, 151, appears much earlier than either the protective antibodies or the flocculating power, after which they run a somewhat parallel course (Horses 193 and 161). It is apparent that, in spite of frequent general agreement between flocculating power and protective action, there remain considerable discrepancies among individual serums which will have to be elucidated by further studies.

DISCUSSION.

Although, at present, it is impossible to give a lucid explanation of the mechanism of the reaction described, it would seem that an immune reaction, exhibiting strict species and a high degree of type specificity may be obtained with substances extracted from the pneumococcus cell by absolute or 95 per cent ethyl alcohol. The method

of preparing the alcoholic antigen does not preclude the possibility that, besides lipoidal substances, certain impurities of protein or carbohydrate character may have been carried over into the extract. The extracted material so far has been obtained only in such small amounts that an exact chemical analysis has not been possible; also, no other solvents have been used. It would seem, however, from the tests done that only traces of substances of protein or carbohydrate nature can have been present in the extract. The reaction apparently depends essentially upon the presence of bacterial lipoids.

In this connection, there comes to mind the work of Avery and Heidelberger (8–10), who, basing their conclusions upon precipitin tests, attribute the type specificity of the pneumococcus to a substance of carbohydrate nature while a nucleoprotein is designated as the carrier of the species specificity of the organism. On the other hand, Zinsser and his associates (11–14) have prepared, from various bacteria, including also the pneumococcus, substances—residue antigens—which are free from coagulable protein and are capable of reacting specifically in a precipitin test with the homologous antiserum. Both of these substances, the polysaccharide of Avery and Heidelberger and the residue antigen of Zinsser, were found by the respective authors to be incapable of producing antibodies. Perlzweig and Steffen (15), in studying different fractions of the pneumococcus for their immunizing effect in mice, found the alcohol-soluble fraction of a trypsin digest of Pneumococcus Type I organisms as active as the ordinary vaccine of heat-killed pneumococci in affording protection, while they failed to obtain immunity with alcohol, ether, chloroform, and acetone extracts made from intact, dried pneumococci. They also state that among the various antigenic solutions which were tested for a precipitation with immune serums, some of the potent alcohol-soluble immunizing antigens failed to react, while, in other instances, precipitation was observed. In this connection, the experiments of Brotzu (16), who found ether-extracted pneumococci to lack antigenic activity in the immunization of rabbits, are of interest.

It remains for further study to coordinate the results obtained with the alcohol-soluble substances described in this paper with those described by the investigators cited above.

Although the type specificity of the reactions is quite marked in some of the tests, more particularly with Type I antigen; in others, it is not quite so definite. Similarly, although the results of the flocculation reactions in general appeared to correspond to those of the protection test, in many instances they were divergent. Since the protection test varies so markedly with the virulence of the

pneumococcus, it is not possible to determine as yet which test yields the more uniform quantitative results. The extent to which differences in the activity of the several antigens and serums figure in the test and whether or not the test can be more accurately standardized for quantitative determination, will require special investigation. Nevertheless, the results obtained so far indicate the desirability of further study of this reaction in connection with the type diagnosis of pneumonia and suggest its value in the standardization of Type I antipneumococcus serum.

SUMMARY AND CONCLUSIONS.

1. A flocculation reaction has been described which occurs between alcoholic extracts of pneumococci and antipneumococcus serum.

2. The reaction appears to be species-specific. It is not strictly type-specific, as slight or moderate cross-reactions occurred between Type I serums and Type II and Type III extracts.

3. The flocculating power of the serum from five horses undergoing immunization with pneumococcus, Type I, did not develop to any extent before the end of the 4th or 5th month.

4. In the case of two of these horses in which it was possible to carry out parallel tests on a larger number of subsequent bleedings until the end of immunization, some relationship was suggested between the flocculating power and the protective titer as ascertained by the routine method of standardization in mice.

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