STUDIES ON PNEUMOCOCCUS IMMUNITY.

III. The Nature of Pneumococcus Antigen.*†

By WILLIAM A. PERLZWEIG, Ph.D., and GUSTAV I. STEFFEN.

(From the Hygienic Laboratory of the United States Public Health Service and the Second Medical (Cornell) Division and Pathological Department of Bellevue Hospital, New York.)

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In the two preceding articles of this series it has been shown by Cecil and Steffen (1) that monkeys may be immunized against pneumococcus pneumonia by subcutaneous or intravenous injections of pneumococcus vaccine. Inasmuch as rather large doses of vaccine were required to obtain complete immunity by subcutaneous injections it seemed desirable to investigate the chemical nature of pneumococcus antigen with the hope that some method might be devised for separating the immunizing fraction from the toxic fraction of the microorganism and thereby provide a more practical agent for prophylactic vaccination against pneumonia in man.

Methods.

Most of the antigens tested in this study were prepared from a highly virulent strain of Pneumococcus Type I. Later on, in order to corroborate the results obtained with Pneumococcus Type I, some experiments were also carried out with Pneumococcus Types II and III.

The experiments were all performed on white mice. The vaccines and antigens were in every instance administered subcutaneously in a volume of 0.5 cc. When two injections of vaccine, or antigen, were administered, an interval of 6 or 7 days elapsed between the two injections. When three or four injections of antigen were given the interval between the injections was 3 or 4 days. The immunity

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of the vaccinated mice was usually tested 9 to 10 days after the last injection of antigen by inoculating them intraperitoneally with various dilutions of a broth culture of the homologous type of pneumococcus. The culture was diluted with plain broth and the volume injected was invariably 0.5 cc. The cultures were carefully controlled by examination of stained smears and agglutination and bile solubility tests. Each experiment was controlled by injecting unvaccinated mice intraperitoneally with one or more lethal doses of pneumococcus culture (0.0000001 and 0.00000001 cc.). All of these control mice, a total of 138, without a single exception, died in less than 72 hours and pneumococci were found in the peritoneal exudate of each of them. In view of the invariably high virulence of the strains employed each survival among the experimental mice must be regarded as significant and showing the production of definite immunity by the antigen in question. A mouse was counted a survival if it lived for 5 days or more after the inoculation of the virulent culture. Usually the mice remained under observation for 7 to 10 days after the inoculation. Each dead mouse was dissected and smears from the peritoneal exudate were examined for pneumococci. In cases of doubt cultures were made on blood agar plates.

I. Immunization with a Saline Suspension of Killed Pneumococcus Type I.

It was first established that small doses of a saline suspension of Pneumococcus Type I, killed by heating at 60°C. for 1 hour, induce immunity in mice against subsequent infection. The importance of giving a small injection of vaccine, followed 1 week later by a larger dose, is demonstrated in the following two experiments.

Experiment 1.—Nine mice received each a single subcutaneous injection of 700 million (0.5 cc.) Pneumococcus Type I saline vaccine. 10 days later they were injected intraperitoneally with a virulent culture of Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.00001	1	2
3	0.0001	3	0

Experiment 2.—Sixteen mice were each injected with 100 million (0.5 cc.) Pneumococcus Type I saline vaccine. 7 days later they received a second dose of 700 million each. 10 days after the last injection of vaccine they were all inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
2	0.000001	0	2
3	0.00001	0	3
3	0.0001	0	3
3	0.001	0	3
3	0.01	0	3
2	0.1	1	1

A moderate immunity was induced by one subcutaneous injection of vaccine (Experiment 1), but a much higher degree of immunity was obtained when two injections were given.

Wadsworth (2) found recently in studying the same question that it took no less than six injections of 6 billion each of killed pneumococci to protect white mice against an infecting dose of 0.001 cc. of a culture of Pneumococcus Type I with about the same virulence as that of the strain used in these experiments. This divergence between the observations of Wadsworth and our own afford some support to the contention of Much (3) that very small initial doses of vaccine are essential to the development of a high degree of immunity.

II. Immunization with the Protein Fraction of the Pneumococcus.

The immunizing properties of the protein fraction of the pneumococcus were studied next. In order to alter the chemical nature of the proteins as little as possible the following methods of separation were used.

(*a*) *Rowland's Method* (*4*).—Moist sediments of living pneumococci were thoroughly mixed with twice their weight of anhydrous sodium sulfate and alternately frozen and thawed at 37°C. for several days. Sufficient water was added to make a saturated solution of sodium sulfate at 37°C. The precipitated protein was washed with additional sodium sulfate solution, redissolved in very dilute (0.01 N) sodium carbonate solution, and freed of the adhering salt by dialysis. As in Rowland's study of the plague bacillus the resulting product was found to be chiefly nucleoprotein as shown by the presence of organic phosphorus and of the purine bases, guanine and adenine, on hydrolysis with nitric acid. The slightly alkaline solution was filtered

through a diatomaceous earth filter (Mandler), tested for sterility, and inoculated into mice to determine its antigenic value. In vaccinating mice with the various fractions and extracts of pneumococcus the dosage was determined by the concentration of the bacterial suspension from which the fraction had been derived; for example, if the original suspension contained 100 billion pneumococci per cc., mice which received 0.1 cc. of the protein fraction were recorded as having received 10 billion pneumococci.

Experiment 3.—Eleven mice each received two subcutaneous doses of an alkaline solution of Pneumococcus Type I protein, corresponding to 200 million and 4 billion pneumococci respectively. 10 days after the second dose, which was given 7 days after the first, the mice were injected intraperitoneally with a broth culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
4	0.0001	0	4
3	0.001	1	2
4	0.01	1	3

(*b*) *Solution of Pneumococci in Bile Salts.*—The property of pneumococci to dissolve in bile and in solutions of bile salts was utilized for the further study of pneumococcus antigen. To saline suspensions of Pneumococcus Type I sodium choleate was added to make a 1 per cent solution, also 0.2 per cent disodium phosphate, and the mixture incubated at 37°C. for 2 to 4 hours, until solution of the pneumococci was complete or nearly so. The undissolved material was separated by centrifugalization and the supernatant fluid thrown into nine volumes of 95 per cent alcohol. The abundant white precipitate was allowed to settle over night, filtered off, and washed free of the bile salts with 90 per cent alcohol. The same nucleoprotein characteristics were found in this substance as in that obtained by Rowland's method. It is soluble in water and in slightly alkaline solutions, coagulated by boiling in the presence of a little acid, contains organic phosphorus and purine bases, and responds to all protein color reactions. A slightly alkaline filtered solution was used in the following experiment.

Experiment 4.—Nine mice were each injected subcutaneously with a protein solution obtained from a bile salt solution of Pneumococcus Type I, the dose corresponding to 2 billion pneumococci. 10 days later the mice were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.00001	1	2
3	0.0001	2	1
3	0.001	2	1

From these experiments it will be seen that the antigen is either a part of the protein fraction of the pneumococcus or else is some substance attached to it.

III. Immunization with Proteolytic and Autolytic Cleavage Products.

Rosenow (5) claims to have shown that autolyzed pneumococci contained an antigen. Rowland (4) showed that his plague bacillus nucleoprotein lost its toxic properties and none of its immunizing properties if permitted to autolyze for as long as 40 days. Jobling and Strouse (6) showed that pneumococcus extracts obtained by digestion with leucoprotease raised the opsonic index in rabbits considerably. Hirschfelder (7) digested cultures of pneumococci with pancreatin for 15 minutes and was able to confer active immunity upon rabbits by injecting the acid filtrates from these digests. So far as the writers are aware Hirschfelder has not reported any further studies along this line since 1912. The above indications that the antigen appears to be resistant to proteolytic ferments raises the time-honored question: Is antigen necessarily of protein nature? It will be remembered that Jacoby's "protein-free" ricin was shown by Osborne, Mendel, and Harris (8) to be albumin-like in nature, when they demonstrated its digestibility with trypsin and consequent loss of antigenic property. In the following experiments it was established that prolonged digestion with trypsin does not destroy the pneumococcus antigen. Protein fractions of pneumococci prepared as described above either by bile salt solution and precipitation with alcohol or by Rowland's sulfate method were digested with trypsin in an alkaline medium, pH 7.7 to 8.0, at 37°C. from 4 to 21 days in

the presence of chloroform. The latter was used for the double purpose of preserving the digest and of removing the effects of the lipoidal antiferments (Jobling and Strouse (6)).[1] The progress of digestion was followed by determinations of non-protein nitrogen by Greenwald's trichloroacetic acid method of precipitation (9) and Folin's micro Kjeldahl method (10). It was found that alkaline or acid filtrates of such digests in which the non-protein nitrogen reached 85 per cent of the total nitrogen retained the original immunizing effect on white mice.

Experiment 5.—A solution of Pneumococcus Type I protein, obtained by dissolving pneumococci in sodium choleate, precipitating with alcohol, and redissolving in distilled water, was digested with trypsin for 4 days and gave a $\frac{\text{Non-protein nitrogen}}{\text{Total nitrogen}}$ ratio of 57 per cent. The total nitrogen in the filtrate was 25.2 mg. per 100 cc., of which 1 cc. represented 40 billion pneumococci. One dose, corresponding to 1 billion pneumococci, was administered subcutaneously to each of six mice, which were inoculated intraperitoneally 10 days later with a virulent Pneumococcus Type I culture.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	2	1
3	0.00001	1	2

Experiment 6.—In this experiment the same solution as above was used but in this instance it was digested for 25 days. The $\frac{\text{Non-protein nitrogen}}{\text{Total nitrogen}}$ ratio in the filtrate was 80 per cent. Six mice were each injected subcutaneously with the equivalent of 2 billion pneumococci. 10 days later they were inoculated intraperitoneally with a culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.00001	1	2

In another experiment a Pneumococcus Type I protein, obtained by Rowland's method, was used. The digestion with trypsin was in this instance continued for 21 days.

[1] Jobling and Strouse (6), p. 862.

Experiment 7.—Ten mice were injected subcutaneously with two doses of this preparation, equivalent to 200 million and 4,000 million respectively of killed pneumococci. The interval between the injections was 7 days. 11 days after the last injection of antigen the mice were inoculated intraperitoneally with a culture of virulent Pneumococcus Type I.

No. of mice.	Culture.	Died.	Survived.
	cc.		
10	0.01	2	8

Autolytic digests of pneumococci were also tested in the same way, with similar results. But the digestion of bacterial protein by autolysis is so much slower than that by proteolytic enzymes that the process cannot be considered advantageous.

Hirschfelder's method of obtaining bacterial antigens was carried out with some modifications. Instead of using cultures for digestion with trypsin, saline suspensions of freshly centrifugalized pneumococci were used. The suspensions were made alkaline with sodium carbonate and sodium phosphate to the reaction of pH 7.6 to 8.0. 0.2 per cent of a 1:1 mixture of trypsin (purified, Parke, Davis and Company) and holadin, an extract of the pancreatic enzymes (Fairchild Brothers and Foster), was added. Holadin was used because of its good lipolytic properties, with the object of lessening the possible antitryptic effects of the bacterial fats and lipoids. Chloroform was also added for the same reason and in addition to act as a preservative. The digestion was permitted to continue for 18 hours at 37°C., the undigested portion was separated by centrifugalization, and the supernatant fluid was carefully acidified with dilute hydrochloric acid until the maximum precipitation of the alkali metaproteins was effected. The precipitate was permitted to settle for several hours, removed by centrifugalization, and the supernatant fluid filtered through a Mandler filter. The resulting filtrate contained only 25 per cent of the original nitrogen of the bacterial suspension, all of which was in the non-coagulable state. Precipitation and color reactions showed that amino-acids, polypeptides, and proteoses were present in this filtrate. The presence of proteoses was shown by precipitation tests with trichloroacetic and picric acids. The presence of amino-acids was ascertained by a positive ninhydrin reaction.

The biuret, Millon, and xanthoproteic reactions were likewise positive. The biuret test was of the pink-violet color, indicative chiefly of the lower peptones and polypeptides. It was possible to obtain with this filtrate a high grade of immunity in mice against Pneumococcus Type I.

Experiment 8.—In this experiment the mice received two subcutaneous doses of antigen 7 days apart. 10 days after the second injection the mice were inoculated intraperitoneally with a virulent Pneumococcus Type I culture as in preceding experiments.

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
140 + 700	0.00001	4	3	1
140 + 1,400	0.00001	6	2	4

In the next experiment larger doses of antigen were employed with excellent results.

Experiment 9.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
140 + 1,400	0.0001	5	0	5
140 + 2,800	0.001	5	0	5

In the following experiment three or four doses of antigen were given 3 to 4 days apart and a still higher grade of immunity was produced in the mice.

Experiment 10.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
175 + 350 + 700	0.001	5	0	5
175 + 350 + 700 + 1,400	0.01	5	2	3
175 + 350 + 700 + 1,400	0.02	4	1	3

A single dose of antigen, even when very large, was not so effective as several smaller doses.

Experiment 11.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
5,600	0.01	3	2	1
5,600	0.05	3	3	0
8,500	0.001	2	2	0
17,000	0.001	2	2	0
25,500	0.001	2	2	0

These experiments show that with several smaller injections of this antigen immunity against as much as 0.02 cc. of culture of virulent Pneumococcus Type I could be obtained. The degree of the immunity can be appreciated by the realization that 0.02 cc. of the culture was equivalent to several hundred thousand lethal doses. The single administration of one comparatively large amount induces practically no protection, as shown in Experiment 11.

In order to "deproteinize" the acid filtrate of the tryptic digest further, a portion of the latter was added to ten volumes of absolute alcohol. The resulting precipitate was collected by centrifugalization, washed in alcohol, and redissolved in physiological salt solution. This solution gave typical proteose reactions but contained no coagulable protein. When administered to mice subcutaneously in two doses it failed to protect them even against small amounts of pneumococcus culture.

Experiment 12.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
100 + 5,000	0.00001	3	3	0
100 + 5,000	0.0001	3	3	0

On the other hand, the alcoholic filtrate, when evaporated in vacuum at 35–40°C. to dryness and redissolved in saline afforded considerable protection to mice, as great as the ordinary vaccine of heat-killed pneumococci.

Experiment 13.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
100 + 5,000	0.001	3	0	3
100 + 2,000 + 10,000	0.01	3	1	2
100 + 2,000 + 10,000	0.1	3	1	2

This antigen gave no cross-immunity against a culture of virulent Pneumococcus Type II.

Experiment 14.—

Dose of antigen in millions of pneumococci.	Culture of Pneumococcus Type II.	No. of mice.	Died.	Survived.
	cc.			
100 + 2,000 + 10,000	0.00001	3	3	0

The alcohol-soluble fraction of the trypsin digest of which only a very small amount was available for chemical study contained but little nitrogen (5 mg. per 100 cc. of solution, 1 cc. of which was equivalent to 80 billion pneumococci), gave a negative biuret test, and positive Millon, xanthoproteic, and ninhydrin tests, showing the presence of aromatic (cyclic) amino-acids, and the absence of higher polypeptides, peptone, and proteoses. It is generally conceded that the amino-acids and lower polypeptides do not act as antigens in any sense. One is forced then to assume that the antigenic factor in the alcohol-water-soluble fraction is a non-protein, non-lipin substance.

That the antigen is not of lipoidal or fatty nature was demonstrated by thoroughly extracting the acid filtrate of the trypsin digest with ether for several days. The alcohol-soluble fraction of the trypsin digest was treated in the same manner. These lipin-free antigen solutions conferred a degree of immunity equal to that of the unextracted antigen solutions.

Experiment 15.—

Dose of antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
100 + 5,000	0.001	3	0	3
100 + 5,000	0.01	3	0	3
100 + 5,000	0.1	3	1	2

In this connection it may be mentioned that alcohol, ether, chloroform, and acetone extracts were made from intact dried pneumococci, and sodium salts were prepared from the fatty acids of pneumococci in accordance with Warden's method (11). Repeated attempts to immunize mice with these fatty and lipoidal fractions failed. These sodium salts of fatty acids obtained from Pneumococcus Type I were dissolved in Ringer's solution and cholesterol was added. Warden found in his experiments that cholesterol reinforces the antigenic value of the bacterial fats.

Experiment 16.—Six mice were each injected subcutaneously with three doses of a solution of a sodium salt of fatty acids from Pneumococcus Type I reinforced with cholesterol. The amount given in each injection was 0.5 cc., containing 0.25 mg. of soap and 1 mg. of cholesterol. At the same time three control mice were injected with three doses of a solution of cholesterol, each dose containing 1 mg. of cholesterol. 9 days after the last injection the vaccinated mice and the control mice were each inoculated intraperitoneally with 0.0000001 cc. of a broth culture of virulent Pneumococcus Type I. All the mice died in 48 hours.

Experiment 17.—Antigen prepared as in the preceding experiment. Feb. 7, 1921. Five mice received each 0.5 cc. of the antigen subcutaneously, containing 1 mg. of sodium soaps from Pneumococcus Type I and 1.25 mg. of cholesterol. The second, third, and fourth doses given subcutaneously on Feb. 10, 12, and 15 respectively contained the same amount of antigen and cholesterol as the first dose. Feb. 23 (8 days after the last injection). The five vaccinated mice and a control mouse were each inoculated intraperitoneally with 0.0000001 cc. of a broth culture of virulent Pneumococcus Type I. All the mice died.

These experiments show that immunity could not be obtained in white mice by subcutaneous injections of salts of fatty acids derived from Pneumococcus Type I even against a very small infecting dose of the homologous organism.

IV. Behavior of Pneumococcus Antigen towards Heat, and Its Keeping Qualities.

Since it was not deemed desirable to introduce preservatives and the uncertainty attendant upon their use with antigen solutions, also in order to test the thermostability of the antigen, the following experiments on the effect of sterilization by heat were performed. Two portions of the acid filtrate of tryptic digests of pneumococci, which contained antigen as shown in Experiment 9, were heated in test-tubes of ½ inch diameter for 5 minutes by immersion in boiling water. The reaction in one tube was kept at its original acidity of approximately pH 6.0, while the contents of the second tube were made alkaline to pH 9.0 with dilute sodium hydroxide. In the acid solution a slight precipitate was produced on heating, whereas the alkaline solution remained clear. After cooling, both solutions were filtered through Mandler filters and clear filtrates were obtained. The acid filtrate gave a negative biuret reaction and contained 21 mg. of nitrogen per 100 cc., while the alkaline filtrate gave a positive biuret reaction and contained 38.5 mg. of nitrogen per 100 cc. Four mice were used for testing each of these two solutions by the method given in preceding experiments.

Experiment 18.—Antigen injections 4 days apart. Inoculation with pneumococcus culture 10 days after last dose of antigen.

Antigen in millions of pneumococci.	Culture.	No. of mice.	Died.	Survived.
	cc.			
Boiled acid antigen.				
100 + 2,000 + 10,000	0.001	4	0	4
Boiled alkaline antigen.				
100 + 2,000 + 10,000	0.001	4	4	0

This experiment shows that boiling the antigen solution for 5 minutes in a slightly acid medium does not destroy its immunizing property, and that boiling the same solution for the same length of time but in alkaline solution appears to destroy the antigen. This single experiment also seems to indicate that the coagulable protein occasionally remaining in the tryptic digest is not the antigenic factor.

In another series of experiments (Experiments 19 and 20) an acid filtrate of a tryptic digest of Pneumococcus Type I and a solution of the alcohol-soluble fraction were used separately for immunization

experiments on mice 1 month after preparation. A portion of each solution with a pH of 6.0 was heated in a water bath at 56°C. for 1 hour. The heated and the unheated solutions were then left in a refrigerator at 4°C. without any preservative for 2½ months and at the end of that period their antigenic power was again tested on mice. In each step of these two experiments the mice received the same quantity of antigen in the same number of doses. Three doses were given 4 days apart. The amounts given were the equivalents of 50, 2,000, and 10,000 million respectively of killed Pneumococcus Type I. 10 days after the last injection of antigen the mice were inoculated intraperitoneally with a culture of virulent Pneumococcus Type I.

Experiment 19.—

Antigen.	Culture.	No. of mice.	Died.	Survived.
	cc.			
Acid filtrate of tryptic digest, kept 1 mo.	0.000001	6	0	6
	0.00001	3	2	1
	0.0001	3	1	2
" " " " " heated at 56°C. for 1 hr. and kept for 2½ mos. after heating.	0.000001	6	2	4
	0.00001	3	0	3
	0.0001	3	1	2
Acid filtrate of tryptic digest, unheated, kept for 3½ mos.	0.000001	5	0	5
	0.00001	3	2	1
	0.0001	3	1	2

Experiment 20.—

Antigen.	Culture.	No. of mice.	Died.	Survived.
	cc.			
Alcohol-soluble fraction of tryptic digest, kept 1 mo.	0.000001	5	0	5
	0.00001	3	2	1
	0.0001	2	2	0
Alcohol-soluble fraction of tryptic digest, heated at 56°C. for 1 hr. and kept for 2½ mos. after heating.	0.000001	5	3	2
	0.00001	3	1	2
	0.0001	3	3	0
Alcohol-soluble fraction of tryptic digest, unheated, kept for 3½ mos.	0.000001	6	3	3
	0.00001	3	2	1
	0.0001	3	1	2

These experiments demonstrate that the slightly acid antigenic solutions may be kept in the refrigerator for at least 3 months and that they may be heated for fully 1 hour at 56°C. without deterioration or decrease in their immunizing property. The question of the duration of the immunity produced in mice by the various antigens is being investigated.

After the procedure for extracting the antigen had been worked out it was observed that it resembled in many respects that of Wildiers' (12) for the preparation of his now classic bios, or growth-promoting factor in yeast, especially as regards resistance to autolytic digestion and solubility in 80 per cent alcohol. This analogy suggested experiments which were carried out with some of the antigenic solutions.

Experiment 21.—0.00000001 cc. (in 1 cc.) of an 18 hour plain broth culture of Pneumococcus Type I was added to each of two tubes containing 10 cc. of plain broth and 0.2 cc. of pneumococcus antigen. Two tubes, each containing 10 cc. of plain broth and 0.2 cc. of 0.9 per cent NaCl were inoculated with 0.00000001 cc. of the culture at the same time and acted as controls. All the tubes were incubated for 6 hours at 37.5°C. At the end of this period the growth was determined by pouring blood agar plates with 1 cc. of the culture and colony count after 18 hours incubation.

Plate No.	Culture and antigen.	Colonies.
1	0.00000001 cc. of culture with 0.2 cc. of antigen.	415
2	0.00000001 " " " " 0.2 " " "	620
3	0.00000001 " " " " 0.2 " " saline.	4
4	0.00000001 " " " " 0.2 " " "	15

The experiment was repeated with the same Pneumococcus Type I antigen with 0.0000001 cc. of a broth culture of Pneumococcus Type II, also with 0.000001 cc. of a broth culture of *Streptococcus hæmolyticus*. In both instances the same remarkable growth-promoting effect was observed. While the number of observations on this phase of our work is still insufficient to warrant definite conclusions, the few experiments described indicate that a non-specific growth-promoting agent for bacteria is extracted along with the antigen. This interesting similarity of two apparently different biological agents may lead to a clearer understanding of the ultimate chemical nature of bacterial antigen. Further research on this aspect of the problem is in progress.

V. Experiments with Antigens Prepared from Pneumococcus Types II and III.

From the experiments which have been presented above it will be seen that water-soluble extracts of pneumococcus antigen can readily be prepared from Pneumococcus Type I by several different methods. It seemed desirable to determine whether similar antigens could be prepared from the other two fixed types of pneumococcus. In the following experiment the antigen was prepared from a virulent Pneumococcus Type II.

Experiment 22.—White mice were injected subcutaneously with a saline suspension of Pneumococcus Type II killed by heating at 60°C. for 1 hour. Six mice were injected subcutaneously each with 100 million (0.5 cc.) Pneumococcus Type II saline vaccine. 6 days later the mice received each 1,000 million (0.5 cc.) Pneumococcus Type II saline vaccine. 11 days after the second injection the six vaccinated mice and two control mice were each inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type II.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.00001	1	2
3	0.001	1	2
1 (control).	0.00000001	1	0
1 (").	0.0000001	1	0

The results of this experiment show that immunity against Pneumococcus Type II can be obtained by two subcutaneous injections of a saline suspension of killed Pneumococcus Type II. In the following experiment an antigen consisting of an acid filtrate of tryptic digest of Pneumococcus Type II was used. The antigen was prepared from a portion of the same suspension of Pneumococcus Type II that was used in Experiment 22.

Experiment 23.—Six mice were each injected subcutaneously with three doses of antigen at 4 day intervals. The amounts given were the equivalents respectively of 100, 2,000, and 10,000 million killed pneumococci. 2 days after the last injection of antigen three of the vaccinated mice and control mice were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type II. The remaining three vaccinated mice and control mice received the virulent culture on the 6th day after the last dose of antigen.

Period after last dose of antigen.	Culture.	No. of mice.	Died.	Survived.
days	*cc.*			
2	0.00001	3	2	1
6	0.0001	3	2	1
Control.	0.00000001	1	1	0
"	0.0000001	1	1	0
"	0.000001	1	1	0

Experiment 24.—In this experiment the alcohol-soluble fraction of a tryptic digest of Pneumococcus Type II was used as an antigen. Six mice received each three doses of this antigen subcutaneously. The injections were given 3 to 4 days apart and the amounts were the equivalents of 100, 2,000, and 10,000 million respectively of killed pneumococci. 6 days after the last injection of the antigen three of the vaccinated mice with controls were inoculated intraperitoneally with a culture of virulent Pneumococcus Type II. The remaining three vaccinated mice were inoculated intraperitoneally with Pneumococcus Type II 11 days after the last dose of antigen.

Period after last dose of antigen.	Culture.	No. of mice.	Died.	Survived.
days	*cc.*			
6	0.00001	3	1	2
11	0.0001	3	2	1
Control.	0.00000001	1	1	0
"	0.0000001	1	1	0

Experiments 23 and 24 show that the filtrates of trypsin digests of Pneumococcus Type II and the alcohol-soluble fraction of these digests confer immunity in mice against Pneumococcus Type II.

Finally, the antigenic power of Pneumococcus Type III was studied. The first experiments with antigens from Pneumococcus Type III were carried out with saline suspensions of killed pneumococci.

Experiment 25.—Twelve mice were each injected subcutaneously with three doses of a saline suspension if Pneumococcus Type III, killed by heating at 60°C. for 1 hour. The injections were given 4 days apart, the doses being 100, 1,000, and 2,000 million respectively. 10 days after the last injection of vaccine the mice and controls were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type III.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.00001	1	2
3	0.0001	1	2
2	0.001	2	0
2 (control).	0.00000001	2	0
2 (").	0.0000001	2	0

This experiment shows that a definite immunity can be obtained against Pneumococcus Type III by three subcutaneous injections of the saline suspension. In the following experiment the antigen used was an alcoholic extract of a trypsin digest of Pneumococcus Type III prepared according to the technique described previously.

Experiment 26.—Six mice were each injected subcutaneously with three doses of an alcoholic extract of a trypsin digest of Pneumococcus Type III antigen. The injections were given at intervals of 4 days and the quantities injected were equivalent to 50, 500, and 5,000 million respectively of killed pneumococci. 7 days after the last injection of antigen the treated mice and control mice were inoculated intraperitoneally with a broth culture of virulent Pneumococcus Type III.

No. of mice.	Culture.	Died.	Survived.
	cc.		
3	0.000001	1	2
3	0.0001	2	1
1 (control).	0.00000001	1	0
1 (").	0.0000001	1	0

As in the case of Pneumococcus Types I and II, so with Pneumococcus Type III, an alcoholic extract of a trypsin digest of the bacteria conferred an immunity against the homologous type, approximately as high as that obtained by the original saline vaccine.

DISCUSSION.

From the experiments which have been reported in this paper it is clear that the immunizing antigen can be isolated from the three fixed types of pneumococcus by comparatively simple chemical methods.

This antigen in its purified form contains only a trace of nitrogen. In the alcoholic extract of a tryptic digest the nitrogen determination showed only 5 mg. of nitrogen per 100 cc. of antigen.

While on the basis of the reported experiments it cannot be definitely stated that the immunizing antigen is non-protein in nature, the evidence presented points strongly in that direction. The solubility in 90 per cent alcohol taken in conjunction with the remarkable heat stability and the resistance to the prolonged action of proteolytic enzymes does establish beyond reasonable doubt that the substance is much simpler in nature than the large and complex molecule of native bacterial protein.

It will be recalled in this connection that the purified pneumococcus antibody obtained recently by Huntoon (13) possessed similar chemical properties. The only notable bacterial antigen possessing properties almost identical with those of our antigen is the "Coagulin B," obtained by Pick (14), which was a saline extract of young cultures of typhoid bacilli. It gave a specific precipitate with homologous immune serum, was protein-free, soluble in alcohol, and resistant to heat and to proteolytic enzymes. This striking parallel came to the attention of the authors after the completion of the experimental work herein described and it served to strengthen their belief in the non-protein nature of the immunizing pneumococcus antigen.

The chemical data pertaining to the constitution of the antigen are mainly negative, resembling in this respect the findings concerning the ultimate composition of two other large classes of biological agents, the ferments and the vitamines. But unlike these last two the chemistry of the bacterial antigens has not been subjected to intensive study.

The relation which the immunizing antigen described in these experiments may bear to the other antigens contained in the pneumococcus, *e.g.* precipitinogen, agglutinogen, and complement fixation antigen, is of obvious interest, and the authors hope to report some interesting data on this subject in the near future. The recent study by Zinsser and Parker (15) renders the further investigation of the relation existing between the various antigens all the more interesting and urgent. It is quite significant that the antigen extracted by Zinsser and Parker from pneumococci by weak alkaline solutions and

deproteinized by acidification and heat yielded a non-protein substance, similar to Pick's "coagulin" (14), which produced specific precipitates with homologous serum, but on the other hand produced no antibodies when injected into animals. It is to be noted that while this substance resembled the antigen described in the present paper in its resistance to heat, it differed from it in being precipitable by alcohol. The immunizing antigen herein described was freely soluble in 90 per cent alcohol and the alcohol-insoluble fraction after careful washing with 90 per cent alcohol contained no immunizing antigen (Experiment 12). These results strongly suggest that the precipitinogen and the immunizing antigen are distinct entities. It may be stated that among the various antigenic solutions tested with immune serum the writers found a number which produced specific precipitates yet failed to immunize mice; on the other hand, some of the potent alcohol-soluble immunizing antigens failed to produce precipitates with homologous serums.

These purified pneumococcus antigens are non-toxic for mice and could probably be used in comparatively large doses in man without exciting severe local or general reactions.

CONCLUSIONS.

1. Active immunity against many lethal doses of Pneumococcus Types I, II, and III may be produced in mice by two or three subcutaneous injections of the homologous type of pneumococcus saline vaccine.

2. Mice may be actively immunized with the protein fraction obtained by treating pneumococci with anhydrous sodium sulfate or by solution of pneumococci in bile salts and precipitation with alcohol. Pneumococcus antigen is therefore carried within or adheres to the protein fraction of the organism.

3. Pneumococcus antigen is resistant to prolonged autolysis and to tryptic digestion, and can be recovered from the soluble portions of digests of either the intact bacteria or the bacterial protein.

4. The antigen may be isolated from each of the three fixed types of pneumococcus by tryptic digestion of the pneumococci and extraction of the digest with 70 to 90 per cent alcohol. The antigen is not soluble in absolute alcohol, nor is it soluble in ether or in the other lipoidal solvents.

5. The immunizing property of slightly acid solutions of the antigen is not impaired by boiling for 5 minutes, nor by heating at 56°C. for 1 hour. Sterile unpreserved solutions of the antigen did not deteriorate by standing in the refrigerator for 3½ months.

6. The exact chemical nature of pneumococcus antigen still remains to be determined. From the experiments reported it appears that the antigen is non-lipoidal, that it probably adheres to the protein fraction in a loose chemical or physical union rather than representing a protein complex of a large molecular size, as shown by its solubility in alcohol, its thermostability, and its resistance to proteolytic digestion.

7. Some of the antigens studied have been shown to contain a non-specific factor promoting the growth of bacteria.

8. The purified pneumococcus antigen solutions are non-toxic for mice.

BIBLIOGRAPHY.

1. Cecil, R. L., and Steffen, G. I., *J. Exp. Med.*, 1921, xxxiv, 245; 1923, xxxviii, 149.
2. Wadsworth, A. B., *J. Immunol.*, 1920, v, 429.
3. Much, H., *Münch. med. Woch.*, 1920, lxvii, 1005.
4. Rowland, S., *J. Hyg.*, 1912, xi, suppl. 1, pp. 11, 20; xii, suppl. 2, pp. 340, 344, 350, 358; 1914, xiii, suppl. 3, pp. 403, 418, 440.
5. Rosenow, E. C., *J. Infect. Dis.*, 1911, ix, 190; 1912, x, 113; 1912, xi, 94, 235, 286; *J. Am. Med. Assn.*, 1918, lxx, 759.
6. Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1912, xvi, 860.
7. Hirschfelder, J. O., *J. Am. Med. Assn.*, 1912, lix, 1373.
8. Osborne, T. B., Mendel, L. B., and Harris, I. F., *Am. J. Physiol.*, 1905, xiv, 259.
9. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.
10. Folin, O., and Farmer, C. J., *J. Biol. Chem.*, 1912, xi, 493.
11. Warden, C. C., *J. Infect. Dis.*, 1918, xxii, 133; xxiii, 504; 1919, xxiv, 285.
12. Wildiers, *La Cellule*, 1900, xvii, 385.
13. Huntoon, F. M., *J. Immunol.*, 1921, vi, 117. Huntoon, F. M., and Etris, S., *J. Immunol.*, 1921, vi, 123. Huntoon, F. M., Masucci, P., and Hannum, E., *J. Immunol.*, 1921, vi, 185.
14. Pick, E. P., *Beitr. chem. Physiol. u. Path.*, 1902, i, 397; also discussed in detail by Paltauf (Paltauf, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 567).
15. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.