CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF A SPECIES-SPECIFIC CARBOHYDRATE OF PNEUMOCOCCI

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The isolation and identification of constituents of bacteria have in many instances disclosed facts of both biological and immunological interest. From investigations of this character the fact has been established that non-protein material of bacterial origin may participate in immunological reactions.

Pick (1) first reported results demonstrating this possibility. He isolated from typhoid bacilli material which failed to give the usual tests for protein but which reacted specifically in antityphoid sera. The residue antigens of Zinsser (2, 3), obtained from a variety of bacteria, were characterized by their non-protein nature. The identification of the type-specific fraction of pneumococci—a non-protein substance—as a polysaccharide established a definite relationship between the chemical constitution and certain biological activities of these organisms (4). Furthermore, the results of investigations based on these chemical studies have defined more clearly the immunological behavior of pneumococci (4). The strict type-specificity of these bacterial sugars in immunological reactions has been established and has been contrasted with the broad species reaction exhibited by pneumococcus protein. Heidelberger's (5) review of the subject contains a complete report of investigations on bacterial polysaccharides. More recently Lancefield (6) has found in hemolytic streptococci a carbohydrate fraction which appears to be a common constituent of these organisms.

The present report contains the results of an investigation of a non-protein substance derived from pneumococci which is chemically and immunologically distinct from both the type-specific capsular polysaccharide and the somatic nucleoprotein. The pneumococcus constituent to be described has been designated Fraction "C," and appears to represent a hitherto unidentified carbohydrate common to the cell body of all R and S forms of pneumococci.

Extraction of the Substance from an R Strain of Pneumococcus

The bacterial cells from 3 liters of broth culture of an R strain derived from Type II Pneumococcus were collected by centrifugation. The bacteria were resuspended in 50 cc. of salt solution and were repeatedly frozen and thawed to break up the bacterial bodies. To this solution of bacteria 0.5 cc. N/1 acetic acid was added, and the mixture was then heated for 10 minutes in a boiling water bath. The tube was cooled and the coagulated protein was separated from the clear supernatant liquid which contained the "C" substance. In this manner the noncoagulable material from 30 to 40 liters of culture was collected. The combined supernatant extract, after neutralization, was finally filtered through a Berkefeld candle and then concentrated *in vacuo* to 50 cc.

TABLE I

Preparation No.	$[\alpha]_D$	Nitrogen	Reducing sugars on hydrolysis	Highest dilution giving a precipitate with Type III antipneumococcus serum*
		per cent	*per cent*	
1†	+22.90	9.40	18.2	1:1,000,000
2	—	5.86	29.5	1:2,000,000
3	+25.0	5.07	30.0	1:2,000,000

* Although Fraction "C" is precipitated by Types I and II antipneumococcus horse sera, Type III serum reacted in highest titre and has been employed as the test serum.

† Preparation 1 was the first preparation made. The material as isolated still gave a faint biuret test. The high percentage of nitrogen in this preparation probably results from accompanying inert proteolytic products.

Preparation of the Substance

The concentrates from 36 liters of bacteria obtained as described above were again acidified with 0.5 cc. of N/1 acetic acid and reheated for 15 minutes at 100°C. A small amount of coagulated protein was thus separated and removed by centrifugation. The clear solution was now treated with 5 volumes of alcohol and, after standing overnight in the icebox, a precipitate settled out which contained all of the so-called "C" substance, together with certain nitrogenous impurities. This precipitate, when redissolved in saline, was found to give a specific precipitin test with antipneumococcus horse serum of Types I, II and III. (A more detailed account of the serological reactions is presented in another part of this report.) The solution of the "C" fraction was found to contain, however, material which gave a positive biuret test, but this impurity was precipitated from solution by making the mixture alkaline with sodium hydroxide, without loss of the serologically reactive substance. The alkaline solution of the "C" fraction, now at a

volume of 50 cc., was reprecipitated by the addition of 5 volumes of alcohol. The material was centrifuged, redissolved in 40 cc. of water and again precipitated from faintly acid solution by alcohol. This procedure was repeated altogether four times. The carbohydrate recovered from the final alcoholic precipitation was dissolved in 15 cc. of water and was cooled to 0°C. To the solution was added 2 cc. of hydrochloric acid (sp. gr. 1.09). A small amount of insoluble inactive material separated from the solution and was centrifuged off. The clear acid solution was now precipitated with five volumes of redistilled alcohol. After standing at 0°C. for 4 hours the "C" substance was separated by centrifugation. It was redissolved in 10 cc. of water and then reprecipitated with alcohol and acid. The final product was washed free from chlorides with 85 per cent alcohol, and was washed finally with redistilled alcohol and ether. The yield was about 65 mg. from 36 liters of broth cultures.

The properties of the various preparations of the "C" substance thus obtained are shown in Table I.

Properties of the "C" Fraction of Pneumococcus

The somatic, species-specific "C" substance as isolated above (Preparations 2 and 3) was found to be an amorphous product soluble in water and insoluble in organic solvents. Its immunological specificity was not impaired by the prolonged action of pepsin at pH 2.0 nor by trypsin at pH 8.1. A solution of the substance (1:300) gave no Millon's test, no biuret test, no xanthoproteic reaction, and no Hopkins-Cole test. Preparation 1 gave a very feeble xanthoproteic reaction, and a very faint ninhydrin test. The solution gave no precipitate with trichloracetic acid, with tungstic acid, with picric acid, nor with sulfosalicylic acid. It did not precipitate on the addition of chloroplatinic acid, copper sulfate, mercury sulfate, nor uranium nitrate. The substance gave a strongly positive Molisch test, and yielded about 30 per cent of reducing sugars on hydrolysis. By treatment with nitrous acid, this substance, like the type-specific polysaccharide of Pneumococcus Type I, was slowly hydrolyzed, with a corresponding loss in immunological activity, and the appearance of reducing sugars. Unfortunately not enough material was available to follow quantitatively the reducing sugars formed, or to determine the phosphorus or sulfur content. Unlike the type-specific polysaccharide of Pneumococcus Type I, the "C" substance contains no amino nitrogen.

On the basis of the chemical data thus far obtained, Fraction "C" appears to be a well defined chemical entity present in pneumococcus cells but distinct from the other fractions. In order to substantiate the validity of this conclusion serological tests were carried out, the results of which may be briefly described as follows: When material prepared according to the method described was mixed with antipneumococcus horse sera of Types I, II and III[1] and incubated, precipitation occurred with each of the three antibacterial sera. A broad reactivity of this character contrasts sharply with the strictly type-specific limitations of the capsular polysaccharides. The tests were repeated employing three different lots of Type I serum, and two of Type II and Type III antisera. In every instance the type-specific polysaccharides reacted only with the homologous antiserum, whereas Fraction "C" was precipitated with each serum, regardless of type. Although these observations are few in number, nevertheless, the results have been sufficiently clear cut to demonstrate serologically the separate identity of the two test substances. Further evidence of this differentiation, apart from the chemical and serological data, may be found in the fact that *non-type-specific R pneumococci contain Fraction "C."* The serological distinction, obtained by the use of antipneumococcus sera of animal origin, has also been observed by employing sera from patients ill with lobar pneumonia. In a separate communication (7), results are presented which demonstrate, during the course of the disease, the independent occurrence of antibodies reactive with three separate constituents of pneumococci.

The "C" substance, on the basis of its broad reactions with antipneumococcus horse sera, is analogous to pneumococcus "nucleoprotein" in that the precipitation of either substance has no type-specific limitations. Since the non-protein Fraction "C" and the so-called nucleoprotein of the cell are so widely different chemically, the similarity in the scope of their serological reactivity indicates that Fraction "C," like the "nucleoprotein," is a common constituent of all pneumococci. Additional evidence in support of this view is brought by the

[1] Types I, II and III antipneumococcus horse sera were obtained through the courtesy of Dr. A. B. Wadsworth from the New York State Health Laborarories, Albany, New York.

fact that a non-protein substance apparently identical with Fraction "C" has been obtained from several strains of both the R and S forms of pneumococci. Material possessing properties similar to the "C" substance has been extracted from two additional R strains—one derived from Type I and the other from Type II Pneumococcus, as well as from Type II and Type III S organisms. These samples were not so highly purified chemically, nor was it determined whether one strain contained more of the substance than another. However, each strain furnished non-protein material which reacted in each of the three types of antipneumococcus horse sera The crude material derived from S cultures by acid and heat extraction contained both the type-specific polysaccharide and the non-type-specific Fraction "C."[2]

In addition to the serological reactions just described a limited number of observations have been made on the toxicity and antigenicity of Fraction "C."

The Toxicity and Antigenicity of Substance "C"

Toxicity.—Mice have been injected intraperitoneally in amounts up to 1 mg. No evidence of toxicity was manifested by the animals nor was purpura produced. For the injection of rabbits preparations of "C" fraction were used which had not been quantitatively analyzed. However, the intravenous injection of 3 cc. of a concentrated extract elicited no toxic symptoms in rabbits.

Antigenicity.—Three rabbits were injected intravenously with concentrated extract. Although the exact content of Fraction "C" was not known, these preparations reacted in high dilutions with antipneumococcus sera. The animals each received 1 cc. of the concentrate daily for 7 days; after a week of rest, daily injections were again given for 7 days; a similar series of injections was repeated a third time. Test bleedings were made on the sixth day after each weekly series of injections. At no time were precipitins for the "C" substance detectable in the sera of the treated rabbits.

[2] After being informed of the above experiments, Drs. Heidelberger and Kendall of the Presbyterian Hospital in this city also encountered the "C" substance as an impurity in their crude Type IV pneumococcus specific polysaccharide, and will shortly publish confirmatory results.

DISCUSSION

The results reported in this communication demonstrate that pneumococci contain a non-protein fraction (Fraction "C") which is distinct from the type-specific polysaccharide. The chemical studies indicate that Fraction "C" does not fall into the category of proteins or their degradation products, but appears to be a nitrogenous polysaccharide analogous in chemical behavior—but not in serological reactivity—to the Type I soluble specific substance. The "C" substance seems to be present in cultures of the organisms in much smaller quantities than the type-specific carbohydrates. The molecule also is probably smaller since it passes through collodion membranes with ease, and through parchment membranes fairly readily. The results of the chemical tests, when taken together with the serological reactions, indicate that Fraction "C" is a common constituent of all pneumococci and apparently is contained within the body of both R and S cells. Chemical analysis and serological reactivity separate it from both the type-specific carbohydrate and the so-called nucleoprotein. In the doses employed, the "C" substance was not toxic for mice or rabbits, nor, under the experimental conditions employed, was it antigenic.

CONCLUSION

Pneumococci contain a non-protein constituent which, on the basis of its chemical and immunological properties, appears to be a carbohydrate distinct from the type-specific carbohydrate and common to the species.

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