THE IMMUNOLOGICAL SPECIFICITY OF STAPHYLOCOCCI

II. The Chemical Nature of the Soluble Specific Substances*

By C. W. WIEGHARD and L. A. JULIANELLE, Ph.D.

(From the Oscar Johnson Institute, Washington University School of Medicine, St. Louis)

(Received for publication, April 1, 1935)

The evidence presented in a previous communication (1) indicates the occurrence of at least two specific types among the staphylococci. While the specificity of the types, however, is occasionally detected by the agglutination reaction, it is demonstrable regularly only by precipitation in homologous antisera, of the soluble specific substances extracted from the organisms. In the preparation of the reactive material a number of methods have been tried, and in a general way, all have been more or less successful with the results varying in quantity of material recovered. It is proposed in the present report to describe the method of extraction and purification finally adopted and to present with this method the chemical properties and characteristics of the reactive substances.

While purified soluble specific substance has been isolated from several different strains of Staphylococcus, the bulk and detail of the study has been conducted with Strains 13 and Mx3. The former is now identified as Type A and was originally isolated from a patient with septicemia. The latter is a Type B strain and it was cultivated from the normal conjunctiva.

Isolation of the Soluble Specific Substances

The bacteria centrifugated in a Sharples from 50 liter lots of 18 to 24 hour broth cultures of staphylococci were suspended in approximately 200 cc. of N/16 hydrochloric acid. Such suspensions were kept in the ice box until the bacteria from 250 liters were accumulated. The suspensions were then extracted by heating in a boiling water bath for 20 minutes. When the mixture had cooled sufficiently to be transferred to 250 cc. bottles it was centrifugated and the clear

* Conducted under a grant from the Commonwealth Fund of New York.

straw-colored supernatant was decanted. At this point a great deal of the inactive material was removed by dropwise addition of 40 per cent alkali (NaOH). The resulting flocculent precipitate was then removed by centrifugation. The supernatant was precipitated again with the strong alkali and separated from the resulting precipitate. This process was repeated until no precipitation resulted from the further addition of alkali. At times, after most of the protein had been precipitated the solution became hazy when more alkali was added, but without the formation of a precipitate. On such occasions, the solution was allowed to stand overnight in the ice box, after which time the coagulated protein was removed.

The clear supernatant (acid to litmus) was treated with two to four volumes of alcohol and about 5 gm. of sodium acetate. The partially precipitated solution was allowed to stand in the ice box overnight. By the next morning a heavy precipitate had settled to the bottom of the flask and most of the alcoholic supernatant was removed by decantation. The precipitate was completely separated from the supernatant by centrifugation. The residue was extracted with 50 to 100 cc. of water (depending on the quantity of precipitate) and any insoluble material was removed by centrifugation. The aqueous extract was precipitated with a solution of 50 per cent trichloracetic acid. After removal of the precipitated material the supernatant was again precipitated with trichloracetic acid and centrifugated. This process was repeated until no precipitation followed the addition of trichloracetic acid. The resulting supernatant was then precipitated with six volumes of alcohol. The very turbid solution was allowed to stand for several days in the refrigerator, after which time the clear alcoholic supernatant was completely decanted from the gummy precipitate which adhered to the walls of the flask. After the last traces of alcohol had been separated from the gummy precipitate by suction it was extracted with a minimum quantity of water (10 to 25 cc.). Any undissolved material was removed and the supernatant was precipitated with trichloracetic acid as described above. The processes of precipitation with alcohol, extraction of the gummy residue with water and subsequent precipitation of this extract with trichloracetic acid were repeated until the aqueous extract no longer contained material precipitable with trichloracetic acid.

The resulting aqueous extract was then diluted to about 100 cc. and decolorized with norit. The clear filtrate was electrodialyzed in a cell similar to that described by Holmes and Elder (2). From time to time a drop or two of concentrated hydrochloric acid was added to the cell and the dialysis was continued until the ammeter reading had remained constant for at least 2 hours after the preceding addition of hydrochloric acid. The final ammeter reading varied from 0.005 to 0.008 amperes. The dialysate was concentrated *in vacuo* to about 10 to 15 cc. and then poured into ten volumes of dry redistilled acetone. After the turbid solution had stood in the refrigerator overnight the precipitated material settled to the bottom of the flask. The clear supernatant was poured off and the gummy precipitate was triturated with dry acetone until it became sufficiently hard and dry to permit effective centrifugation. The now granular precipitate was washed three or four times with absolute alcohol and finally with ether. The yield varied

from about 0.025 to 0.05 gm. This yield does not include, however, the material extracted from waste residues and supernatants which were extracted separately.

Chemical Properties of the Soluble Specific Substances

The soluble specific substances of both Types A and B obtained by the procedure described above are white amorphous powders, readily soluble in water, giving crystal clear aqueous solutions in concentrations of 1 per cent. The substances possess acid properties, since aqueous solutions give an acid reaction to litmus. The Molisch reaction is positive in high dilutions and Fehling's solution is not reduced. When the substances are boiled with mineral acid, however, they are slowly hydrolyzed with the formation of reducing sugars which have not been identified completely as yet. With the chemical change accompanying hydrolysis, the soluble specific substances simultaneously lose their serological reactivity. The substances give no color with iodine-potassium iodide solution. Dilute solutions (1:200) give yellow crystals of iodoform with sodium hypoiodite.

1 per cent solutions of the soluble specific substances do not give the usual color or precipitation reactions characteristic of proteins. Such tests as Millon's, xanthoproteic, Hopkins-Cole and ninhydrin tests are negative. The biuret reaction also may be considered negative, since the color and intensity of the resulting solution match those of a dilute solution of egg albumin (1:2000), which is about the limit of delicacy of the test. Solutions of the substances are not precipitated by the salts of heavy metals (*e.g.* mercuric chloride, silver nitrate or neutral lead acetate), by acids (*e.g.* trichloracetic acid, picric acid, sulfosalicylic acid and potassium ferrocyanide and acetic acid) or by concentrated solutions of salts such as ammonium sulfate, sodium sulfate, sodium chloride, etc. Uranyl nitrate does not cause immediate precipitation but if the solution is allowed to stand for several days a yellow precipitate is obtained.

While the Type A and Type B soluble specific substances probably do not represent single, chemically pure compounds, it seems, nevertheless, that the major portion of the impurities have been removed. On the basis of the properties described above, it is reasonable to conclude that the serum reactive substances are polysaccharides. The chemical data bearing on the nature of both soluble specific substances are recorded in Table I and they indicate the constancy of composition of various preparations.

In studying the properties submitted in Table I, it is obvious that some of the data merit comment. The values for carbon are low since the theoretical value for a polysaccharide of formula $(C_6H_{10}O_5)_x$ is 44 per cent. The consistently high percentage of phosphorus which appears to be due neither to inorganic phosphate nor protein

(as shown above) suggests the possibility that the polysaccharides may be phosphoric acid derivatives. In this connection it is interesting to note that the calculated values for carbon and phosphorus for a monophosphate of a disaccharide are 33 and 7 per cent respectively. It is possible that the polysaccharides may be complexes in which the same ratio, of two simple hexose units to one of phosphoric acid, is present. Although the polysaccharides are apparently protein-free it has not been possible as yet to isolate a preparation

TABLE I

Chemical Characteristics of the Specific Carbohydrates of Types A and B Staphylococcus

	Type A*		Type B*	
Ash, *per cent*	1.8		3.5	
Carbon,† *per cent*	34.70		36.12	
Hydrogen, *per cent*	6.41		6.58	
Nitrogen, *per cent*	4.09		3.84	
Phosphorus, *per cent*	6.27		6.40	
Neutral equivalent N/50 NaOH	776		806	
Optical rotation $[\alpha]_D^{32°}$	+6.7°	+7.57°	+69.4°	+65.4°
Total reducing sugar, ‡*per cent*	26.1	24.0	38.84	36.7
Fermentable sugar, *per cent*	1.78	1.4	35.01	33.3
Non-fermentable sugars, *per cent*	24.32	22.6	3.87	3.4

* Both substances reacted in homologous immune sera to a dilution of 1:6,000,000.

† All percentages are calculated on ash-free basis.

‡ Shaffer-Hartmann procedure used for determining the reducing sugars. All values are calculated as glucose.

completely free of nitrogen. Whether this nitrogen is an impurity or an integer of constitution as Avery, Goebel and Heidelberger (3) have shown for Type I Pneumococcus must await future work. The optical rotations of the two preparations are given for each type and as can be seen these values are almost identical; likewise, the quantity of reducing sugars are of the same order of magnitude. The similar chemical composition of the two polysaccharides is indeed as impressive as the difference in the serological reactivity. On the basis of the carbon, hydrogen, nitrogen and phosphorus content as well as the

neutral equivalents, it would seem that the two polysaccharides are empirically strikingly similar.

The Hydrolytic Products of the Soluble Specific Substances

As a preliminary approach to a study of the hydrolytic products of the type polysaccharides, observations were made on the rotation between time of hydrolysis, alterations in optical rotation and loss in serological specificity of the carbohydrates. For this purpose, 0.2322 gm. of Type A and 0.2281 gm. of Type B polysaccharides were dissolved in 10 cc. of normal sulfuric acid. The optical rotations and precipitation in homologous immune sera were recorded, and the solutions were then hydrolyzed in sealed tubes in a boiling water

TABLE II

Relation of Serological Specificity and Optical Rotation to Acid Hydrolysis

Period of boiling	Type A carbohydrate		Type B carbohydrate	
	Optical rotation $[\alpha]_D^{25°}$	Serological activity	Optical rotation $[\alpha]_D^{25°}$	Serological activity
0	+6.7°	++++	+69.4°	++++
2 hrs.	19.1°	+	29.5°	++
4 hrs.	19.6°	−	22.1°	−
5½ hrs.	21.2°*	−	23.1°*	−

* If calculated on the basis of reducing sugars, the optical rotation for Type A = +81.1°, for Type B = +59.7°.

bath. At definite intervals, readings were made on the serological titres and optical rotations, the latter figures calculated on the basis of weight of polysaccharide. The results of this experiment are presented in Table II. Examination of this protocol reveals that serological specificity is greatly diminished within 2 hours, and that it is destroyed completely within 3 hours. Similarly, the alterations in optical rotation indicate an increasing hydrolysis up to about 5 hours when the polysaccharides appear to be totally hydrolyzed.

Preparation of Osazones from the Products of Hydrolysis

The solutions from the hydrolyses described in the above paragraph were used for the preparation of the osazones. The solutions were

diluted to about 50 cc. and neutralized with barium carbonate. The barium sulfate was then filtered off. The filtrate was treated with a very small quantity of norit and an excess of calcium carbonate. After the mixture had been heated to boiling, it was filtered and the resulting filtrate was concentrated *in vacuo*. The residue was extracted three times with 10 cc. of methyl alcohol for each extraction. The alcoholic extracts were filtered and concentrated *in vacuo* and the residue was taken up in about 10 cc. of water. The aqueous extract was filtered and then treated with 0.25 gm. of a mixture consisting of two parts recrystallized phenylhydrazine hydrochloride and three parts of sodium acetate.

Characteristics of Osazone Derived from Type A Carbohydrate.—Immediately after the phenylhydrazine mixture had completely dissolved, the solution became hazy. After shaking, a yellow feathery precipitate began to settle out. The mixture was allowed to stand at room temperature for several hours after which it was filtered. An attempt was made to recrystallize the material but without success. The filtrate was heated in a water bath for 2 hours and the clear solution was allowed to cool very slowly. The crystals which had settled out were examined under the microscope, and they were found to consist of rosettes of ferny petals. The crystals were filtered off and washed with alcohol. The melting point of the crystals was 195°.

More phenylhydrazine acetate mixture was added to the filtrate and the resulting mixture was heated in the water bath. No more crystals were obtained.

A second portion of Type A was hydrolyzed and treated as above except that the yellow precipitate obtained on the addition of the phenylhydrazine mixture was not removed. The entire mixture was heated for 2 hours in the water bath. The same type of crystals were obtained with a melting point of 195°.

The information gained on the osazone derived from Type A polysaccharide fails to identify the simple sugar resulting from hydrolysis of the original carbohydrate.

Characteristics of Osazone Derived from Type B Carbohydrate.—The solution obtained by treating the simple sugar with phenylhydrazine acetate mixture was perfectly clear even after standing several hours at room temperature. After the mixture was heated

on the boiling water bath for an hour, the resulting crystals were filtered off. A second fraction was obtained by heating the solution further. The crystals were combined and recrystallized from 60 per cent ethyl alcohol. The melting point was 204°. A mixed melting point with glucosazone showed no depression.

The melting point and crystalline structure of the osazone from Type B are identical with those of glucosazone. The identification of glucosazone limits the sugar formed on hydrolysis of the specific substance to glucose or its amine, fructose or mannose. Since the hydrolyzed material does not give a Seliwanoff reaction and it does not yield an insoluble hydrazone, it appears likely that the simple sugar of Type B is glucose. Conclusive identification of glucose, however, is lacking at present, but with accumulation of material it is planned to oxidize the sugar to the corresponding acid thus making it possible to arrive at its identification through its potassium salt.

Differences in the nature of the simple sugars of the two specific carbohydrates are also brought out in the quantitative determination of the reducing sugars as will be seen on restudy of Table I. The Shaffer-Hartmann procedure was used for the determination of reducing sugars, and all values are calculated as glucose. It is interesting that the sugars resulting from hydrolysis of Type A polysaccharides are chiefly non-fermentable while those of Type B are fermentable. Since the phloroglucinol and orcinol reactions are negative it would seem that the non-fermentable sugar of Type A is not due to pentoses or uronic acids. Since qualitative tests are at their best extremely unreliable, no interpretation of the quantitative results is attempted at this time.

DISCUSSION

That the two soluble specific substances of Staphylococcus are immunologically distinct has been shown in a previous report (1). It was perhaps to be expected on the basis of different serological reactivity that the two substances were also chemically different. The observations reported in this communication give experimental verification of this assumption. While both substances have not been recovered free of nitrogen, it is, nevertheless, obvious that they are essentially carbohydrates and that their serological specificity disappears as hydrolysis of the soluble specific substance proceeds.

The chemical differences between Type A and Type B carbohydrates have been demonstrated in this report. The specific rotation of Type A is +6.7 as compared with +69.4 for Type B. Following hydrolysis, Type A yields 26.1 per cent reducing sugars of which 1.78 per cent is fermentable and 24.32 per cent is non-fermentable. Type B on the other hand yields 38.84 per cent reducing sugars of which 35.01 per cent is fermentable and 3.87 per cent is non-fermentable. Moreover, the simple sugar of Type B seems to be glucose while that of Type A remains undetermined but suggests, because of its crystalline formation, a different structure.

SUMMARY AND CONCLUSIONS

1. Two carbohydrates have been extracted from different strains of Staphylococcus which are immunologically and chemically distinct.

2. The chemical differences between the two types are manifested principally in optical rotation, and in the simple sugars resulting from hydrolytic cleavage of the specific carbohydrates.

3. The immunological specificity of both polysaccharides is dissipated as hydrolysis proceeds.

4. The results of the chemical reactions reported are discussed.

BIBLIOGRAPHY

1. Julianelle, L. A., and Wieghard, C. W., *J. Exp. Med.*, 1935, **62**, 11.
2. Holmes, H. N., and Elder, A. L., *J. Phys. Chem.*, 1931, **35**, 1351.
3. Avery, O. T., Goebel, W. F., and Heidelberger, M., *J. Exp. Med.*, 1925, **42**, 727.