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Immunological Studies of Polysaccharides of Encapsulated Bacillus. I. Isolation from Smooth *B. rhinoscleromatis.*

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It has been conclusively proved that the specific polysaccharide of pneumococcus possesses antigenic power. It is generally thought that similar substances obtained from other bacteria were devoid of any antigenic property. In the present study it will be shown that the substance prepared from *Bacillus rhinoscleromatis* is antigenic.

The organism was isolated at the Peiping Union Medical College Hospital from the infected submaxillary lymph gland of a clinically established case of rhinoscleroma infection. The cultural characteristics and biochemical reactions of this organism are similar to those described by Morris and Julianelle[1] for 10 strains of *Klebsiella rhinoscleromatis.* The organisms were grown on meat infusion agar, pH 7.6, for 48 hours at 37°C. after which they were washed with distilled water and precipitated by the addition of 95% alcohol. After standing in the ice chest over night the organisms were dried over a boiling water bath and then resuspended in distilled water in the proportion of one part of dried bacilli to 150 parts of water. The flask containing the organisms was thoroughly shaken and placed in the ice chest overnight. At the end of that time glacial acetic acid was added to the gelatinous suspension to effect a concentration of one percent. The flask was placed in a boiling water bath for 30 minutes. It is interesting to note that there was a clearing of the suspension after 15 minutes of heating as all the cells had settled to the bottom of the flask. At this point the flask was agitated and hydrolysis continued. After being allowed to cool, the clear yellowish solution obtained from centrifugation was precipitated with 4 volumes of 95% alcohol to which was added one percent sodium acetate. A fine, white, granular precipitate was observed. Upon standing for several hours the precipitate was collected by centrifugation and dissolved in a small volume of distilled water, centrifuged to remove insoluble substance and reprecipitated with 95% alcohol. Such a procedure was repeated until a perfectly water soluble substance was obtained, involving about 6 precipitations. It might be stated that a second treatment of the

[1] Morris, M. C., and Julianelle, L. A., *J. Inf. Dis.*, 1934, **55**, 150.

remaining intact cells with 1% acetic acid yielded practically no substance of the nature described above. The polysaccharide obtained will be designated as Fraction SI.

The remaining organisms were resuspended in distilled water and 10% potassium hydroxide solution was added to effect a concentration of 0.5%. After a few minutes in the boiling water bath there was a clearing of the suspension indicating complete cell solution. Heating was carried out for half an hour and after acidifying the solution with acetic acid to remove nucleoprotein, it was centrifuged and clarified through a Seitz filter. The solution was treated in the same manner as that described above. The polysaccharide appeared as a flocculent, gelatinous substance in contrast to the finely granular substance of the portion prepared by acetic acid hydrolysis. This polysaccharide will be designated as Fraction SII. A third polysaccharide was prepared from whole organisms by means of the alkaline method and will be designated as Fraction SIII. This last fraction was included for comparative purposes, since this is a common method employed by other workers in the preparation of bacterial polysaccharide of the Friedlander group.

Immune serum against the whole organism was prepared by the inoculation of rabbits intravenously with a heat-killed culture on 3 successive days followed by 4 days of rest; the whole course of injections was repeated twice making a total of 9 injections. Animals were bled 7 days after the last injection. If the titre of the serum was not sufficiently high as measured by the precipitation test with the polysaccharide a few more injections were given.

The 3 fractions obtained by the methods described above were light colored powders easily soluble in saline solution in the concentration of 0.1%; all contained varying amounts of nitrogen although the usual protein tests were negative with 1% solutions, gave a strong Molisch test, did not reduce Fehling's solution except after prolonged acid hydrolysis. Serologically they were indistinguishable at dilutions between 4,000,000 and 6,000,000, all gave precipitation with immune serum, and, at dilution 1:32,000,000, complement fixation with immune serum diluted 1-10.

By the same method employed for preparing immune serum against whole organisms rabbits were immunized with 5 and 10 mg. respectively of the two polysaccharides in order to study their antigenic properties. Five days after the last injection the animals were bled and the sera were tested for complement fixation antibodies. The results are summarized in Table I.

It might be stated that the precipitation test was uniformly nega-

TABLE I.
Complement Fixation Reactions Between Immune Polysaccharide Sera and Fractions SI and SII.

Serum	Total mg. used for immuniz.	Test polys. fraction	Dilution of polysaccharide 1:1,000	1:5,000	1:10,000	1:50,000	1:100,000
S I	10	SI	4+	4+	4+	2+	—
SII	10	SI	3+	—	—	—	—
S I	10	SII	4+	3	—	—	—
SII	10	SII	4+	—	—	—	—

All sera diluted 1:5 in the test.

tive. For simplicity the results obtained with 5 mg. were omitted in the table since they were identical with those of 10 mg. From the table it is quite evident that Fraction SI is weakly antigenic while Fraction SII is insignificant in this respect. Furthermore its action is quite specific as shown by the fact that the titre for the homologous antigen is 10 times higher than that of the heterologous one. Complement-fixation with a suspension of the smooth organism served to substantiate the presence of antibodies. Positive result by complement fixation with anti-Fraction SI serum diluted 1:5 was obtained with a 1:200 dilution of a 24-hour-old agar slant culture (killed at 56°C. for one hour), while only the 1:10 dilution of the same suspension of the organisms showed a positive reaction with the anti-Fraction SII serum. A second series of injections did not improve the titre of the above sera. As will be shown in another paper of this series[2] Fraction SI prepared by acid hydrolysis is sensitizing while Fraction SIII prepared by alkaline hydrolysis is not.

The effect of alkali on the antigenicity of Fraction SI prepared by acid hydrolysis was studied in the following experiment: Part of the polysaccharide was treated with a half percent potassium hydroxide solution and boiled for 30 minutes. Fraction SI so treated with alkali was recovered by 3 precipitations in 95% alcohol. Rabbits were immunized with 10 mg. of this substance in the same manner as that described above. No demonstrable complement fixing antibodies in the sera of rabbits so immunized were found. This finding suggests that Fraction SI is the parent polysaccharide from which Fraction SII might be derived. If it were possible to convert the non-antigenic Fraction SII into the antigenic Fraction SI by boiling in acetic acid such a contention might be substantiated. Some data indicating that such is a possibility were obtained. Experiments along this line are in progress.

[2] Kurotchkin, T. J., and Wong, S. C., in press.

Some of the properties of the polysaccharides may be summarized in Table II.

TABLE II.
Chemical and Serological Properties of Polysaccharide Fractions SI, SII and SIII.

Polys. fract.	% nitrogen	Ppt. titre	Complement fixation titre	Molisch test	Immunological properties
SI	1.5	1:4,000,000	1:32,000,000	Positive	Antigenic sensitizing
SII and SIII	1.5	1:4,000,000	1:32,000,000	"	Negative

Demonstration of the antigenic power of polysaccharides in rabbits by means of the complement-fixation reaction has been recorded by Chow[3] for a polysaccharide prepared from Pneumococcus Type I and by Nishimura[4] for inulin, soluble starch and dextrine; the precipitation test was negative. In this paper we have shown that the same may be true of one of the polysaccharide fractions of *B. rhinoscleromatis*. The result of this experiment suggests that the method employed for isolation of the polysaccharides may be of importance in determining its antigenicity.

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Immunological Studies of Polysaccharides of Encapsulated Bacillus. II. Isolation from Rough *B. rhinoscleromatis*.

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It has been claimed by Julianelle[1] that the R-form of Friedlander bacilli does not elaborate any soluble specific polysaccharide and as a result of this fact the organism is only species specific. It appears of interest to us to extend similar investigation to other closely related species of this group with the object of analyzing the antigenic structure of these strains.

Dissociation of the parent strain, *Bacillus rhinoscleromatis*, was carried out according to the method of Julianelle.[1] Rough variants began to appear as small non-mucoid colonies on the eleventh daily

3 Chow, B., *Chinese J. Physiol.*, 1937, **11**, 223.

4 Nishimura, S., *J. Exp. Med.*, 1929, **50**, 419.

1 Julianelle, L. A., *J. Exp. Med.*, 1926, **44**, 683.