ABSENCE OF A PROSTHETIC GROUP IN A TYPE-SPECIFIC POLYSACCHARIDE OF PNEUMOCOCCUS*

By MICHAEL HEIDELBERGER, Ph.D., COLIN M. MacLEOD, M.D., HAROLD MARKOWITZ, and MARIE M. DiLAPI

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Department of Microbiology, New York University College of Medicine, New York)

(Received for publication, June 15, 1951)

Although the specific polysaccharides of Type II and Type III pneumococcus have been obtained free from nitrogen (1) and/or phosphorus (2) and the repeating structural unit of the Type III substance has been shown to consist only of glucose and glucuronic acid (1, 3) combined as cellobiuronic acid (4), it is a difficult matter to separate the polysaccharides from the last per cent or two of nitrogen- and phosphorus-containing impurities. To some, this has seemed adequate evidence upon which to postulate that the pneumococcal polysaccharides owe their characteristic immunological properties to a prosthetic group (5).

The experiments reported herein were undertaken to determine whether or not a pneumococcal polysaccharide could be purified to the extent of removal of all but traces of nitrogen and phosphorus and yet retain its antigenic properties as indicated by the precipitation of the maximum quantity of antibody in an antiserum formed in the rabbit (2) and as shown by its stimulation of a typical antibody response in the human subject (6, 7).

EXPERIMENTAL

Materials and Methods.—The specific polysaccharide of Type III pneumococcus, S III, was prepared from 16 liters of autolyzed culture in a partially defined medium (8) which had been intermittently neutralized with alkali to increase growth. Owing to the increased yield of specific polysaccharide, 5.95 gm., as determined on an aliquot portion in the region of antibody excess with a calibrated antipneumococcus Type III rabbit serum according to reference 9, the culture was not concentrated *in vacuo*, as recommended in reference 2, but was instead treated with 80 gm. of crystalline sodium acetate per liter and then precipitated directly with 16 liters of ethanol. About 10 per cent of the S III escaped precipitation and was discarded. The remainder was reprecipitated several times with ethanol and isopropanol (10), deproteinized by repeated shaking with chloroform and butanol (2, 11), and precipitated as the barium salt with $Ba(OAc)_2$ at pH 6.6 (2). After reconversion to the sodium salt

* The work reported in this communication was carried out in part under the Harkness Research Fund of the Presbyterian Hospital, New York, and in part under a grant from the United States Army Epidemiological Commission.

(2) and three or four repetitions of this cycle the sodium salt was precipitated twice by 2 to 2.5 volumes of warm saturated Na_2SO_4 solution. After several reprecipitations with neutralized sodium acetate and isopropanol and several centrifugations at 10,000 R.P.M. in the chilled rotor of a Servall centrifuge to remove residual turbidity the solution was divided into two parts and chilled. One (S III_A) was precipitated with 1.5 volumes of chilled redistilled ethanol, the other (S III_B) with about 1 volume of chilled propionic acid (12). S III_B was immediately centrifuged in the cold and washed with chilled redistilled ethanol and redistilled acetone. The properties of the fractions are given in Table I. Nitrogen was estimated by Russell's modification (13) of the Van Slyke-Hiller method (14), phosphorus by that of Fiske and SubbaRow (15).

TABLE I

Properties of S III Fractions Purified by Numerous Repetitions of Mild Procedures

Preparation	Ash as Na	$[\alpha]_D^*$	N*	P*	$\eta_{rel.}$, 0.1 per cent in 0.9 per cent NaCl, 20°C.	Antibody N precipitated from 1.0 ml. rabbit anti-Pn III serum B-58, 68, C-absorbed, 13.5 → 50, by 0.124 mg. S III
	per cent	*degrees*	*per cent*	*per cent*		*mg.*
S III_1‡	6.8	−38	0.13	0.08	2.73	1.346§
S III_2‡	7.3	−46	0.30	0.10	2.70	
S III_A	6.6	−37	0.06	0.01	2.54	1.244
S III_B	3.7	−37	0.05	0.01	2.43	1.250
S III 114A‖		−20	1.4	0.45¶	1.07	
S III 186‖		−34	1.9	0.05¶	1.63	

* Calculated to the ash-free basis.

‡ Two fractions combined and further purified.

§ 0.126 mg. S III used.

‖ Manufactured by E. R. Squibb & Sons, New Brunswick, New Jersey. Analytical data by courtesy of Dr. John W. Palmer.

¶ Calculated from nucleic acid content.

Immunization of Volunteers.—Five subjects, Nos. 96 to 100, were bled and their sera labeled with the subnumeral 0. They then received, subcutaneously, within 48 hours 0.3 and 0.7 ml. of a solution of S III_B in 0.5 per cent phenol-saline containing 50 μg. of S III per ml. (*cf.* reference 16). After about 2 months the subjects were again bled and the sera labelled bleeding sub-1. Analyses for anti-C and antibodies to S III were carried out as described in references 16 and 17.

DISCUSSION

By repeated reprecipitations with ethyl alcohol and isopropyl alcohol, several precipitations as the water-insoluble barium salt, and several saltings out with sodium sulfate at 35–37°C., all as mild procedures as could be applied, a preparation of the specific polysaccharide of Type III pneumococcus has been obtained with as little as 0.05 per cent of nitrogen and 0.01 per cent of phosphorus. It is clear from Table I that this product precipitates almost as much

antibody nitrogen from a C-absorbed (2, 18) Type III antipneumococcus rabbit serum as does a less highly purified sample from which it was, in part, prepared. An even more sensitive indicator of degradation is the relative viscosity, $\eta_{rel.}$, which, also, is almost as high as that of the cruder fraction from which it was prepared, and which compares well with the viscosities of earlier lots of S III isolated from broth cultures (2). It is therefore evident that most of the nitrogen and phosphorus can be removed from S III without subjecting the polysaccharide to serious degradation.

TABLE II

Antibody Response of Human Subjects to Injections of 50 to 70 μg. of Type III Pneumococcus Specific Polysaccharide

Micrograms of antibody N per 4 ml. of serum.

Subject No.	Antibody to: C	S III	Subject Nos.	Range of anti-III values after injection	Mean anti-III values
96_0	5	0	83–95		
96_1	13	21	(11 persons)	2–59	20
97_0	69	0	10, 52–82		
97_1	71	11	(7 persons)	0–99	28
98_0	48	0	201–206	2–29	13
98_1	57	96	(6 persons)		
99_0	39	1			
99_1	34	27			
100_0	7	9			
100_1	7	42			
Mean value after injection..		39			

It now remained to be determined whether or not the new preparation would prove to be fully antigenic in man. In Table II, the antibody response is shown of five human volunteers who received subcutaneous injections of 50 μg. of the new preparation. For comparison the formation of anti-S III in three previous series (6, 7) is given. These subjects were injected with 50 to 70 μg. of S III manufactured by E. R. Squibb & Sons, one lot of which contained 1.9 per cent of N, the other, 1.4 per cent (Table I). In these instances the injected solution contained five other type-specific pneumococcal polysaccharides as well.

Subjects 96 to 100, injected with the material of low nitrogen and phosphorus content, gave the best average response of the four series, so that it is

evident that the elements N and P have nothing to do with the antigenicity of the specific polysaccharide.

The conclusion therefore appears inescapable that the specific polysaccharide of Type III pneumococcus exerts its characteristic immunological properties without benefit of a prosthetic group. Earlier experimental evidence (1–4) against the assumption (5) that such a group is a necessary portion of immunologically specific polysaccharides in general is thus strengthened.

Note Added to Proof.—Professor Maurice Stacey has kindly permitted the authors to record here his substantial agreement with the above conclusions.

SUMMARY

The specific polysaccharide of Type III pneumococcus, with only 0.05 per cent of N and 0.01 per cent of P, still shows almost maximal precipitation of a Type III antipneumococcus rabbit serum and the expected antigenicity in man. There is therefore no evidence that a prosthetic group is involved in these characteristic activities.

BIBLIOGRAPHY

1. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, **42,** 727.
2. Heidelberger, M., Kendall, F. E., and Scherp, H. W., *J. Exp. Med.*, 1936, **64,** 559.
3. Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, 1926, **70,** 613; 1927, **74,** 613.
4. Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, 1937, **121,** 195.
5. (*a*) Stacey, M., *Advances Carbohydrate Chem.*, 1946, **2,** 185. (*b*) Record, B. R., and Stacey, M., *J. Chem. Soc.*, 1948, 1561.
6. Heidelberger, M., MacLeod, C. M., and Di Lapi, M. M., *J. Exp. Med.*, 1948, **88,** 369.
7. Heidelberger, M., Di Lapi, M. M., Siegel, M., and Walter, A. W., *J. Immunol.*, 1950, **65,** 535.
8. Adams, M. H., and Roe, A. S., *J. Bact.*, 1945, **49,** 401.
9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55,** 555.
10. Heidelberger, M., MacLeod, C. M., Markowitz, H., and Roe, A. S., *J. Exp. Med.*, 1950, **91,** 341.
11. Sevag, M. G., *Biochem. Z.*, 1934, **273,** 419.
12. MacPherson, C. F. C., Heidelberger, M., Alexander, H. E., and Leidy, G., *J. Immunol.*, 1946, **52,** 207.
13. Russell, J., *J. Biol. Chem.*, 1944, **156,** 457.
14. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, 1933, **102,** 499.
15. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66,** 375.
16. Heidelberger, M., MacLeod, C. M., Kaiser, S. J., and Robinson, B., *J. Exp. Med.*, 1946, **83,** 303.
17. Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, **97,** 405; **98,** 63.
18. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1937, **65,** 647.