ACTIVE IMMUNIZATION OF MICE WITH THE POLYSACCHARIDES OF PNEUMOCOCCI TYPES I, II AND III

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A great deal of contradictory evidence has been reported in the study of the polysaccharides of the pneumococci as antigens. Avery and Morgan (1) and Avery and Heidelberger (2) failed to induce antibody formation in animals injected with their purified polysaccharides. Schiemann and his coworkers (3–5) obtained a carbohydrate from the pneumococcus cells by a different method from that used by Heidelberger and Avery, but believed that the substance obtained was identical with that of the last mentioned authors. With this substance they were able to produce active type-specific immunity in mice, although no precipitins were found in rabbits so treated. Enders (6) has obtained a distinct polysaccharide (substance A) from pneumococci Type I, and with this substance was able to produce typical anaphylactic shock in guinea pigs passively sensitized with serum containing only antibody against this A substance. The characteristic behavior of this substance A is that its activity both *in vitro* and as an immunizing antigen is greatly diminished or lost by heating in a weak alkaline solution. Wadsworth and Brown (7) reported the isolation of a specific substance from the Pneumococcus Type I cell disrupted by repeated freezing and thawing. This substance, of carbohydrate nature, precipitates specifically its homologous type serum in a high dilution. With the same serum, after the antibodies against the specific carbohydrate of Heidelberger and Avery, prepared from culture filtrates, had been absorbed, ten times the amount was required for precipitation. This substance injected into mice in the same amounts used by Schiemann, immunized them against Pneumococcus Type I virulent culture.

Zozaya (8) has reported work on the production of active immunity in mice, rabbits and horses with the specific polysaccharides of the three types of pneumococci adsorbed on colloids. In the present study we are reporting further work on the active immunity produced in mice by the injection of the three specific polysaccharides of the pneumococci, adsorbed on colloids and alone.

Method

Preparation of Colloid Carrier and Adsorption of Polysaccharide on Collodion Particles and Carbon.—The methods used for the preparation of the collodion carrier and adsorption of the polysaccharides were the same described in a previous communication (8). The adsorbed collodion particles were rid of free polysaccharide by washing unless otherwise stated, and standardized so that their turbidity would equal that of a suspension of *B. coli* of 25,000,000,000 per cc. The suspension of collodion particles upon which the polysaccharides have been adsorbed will be designated merely as collodion particles of whatever polysaccharide was used for the adsorption.

The adsorption of polysaccharide on carbon and the determination of the amount of polysaccharide irreversibly adsorbed have been described in a previous study (9). The suspensions of adsorbed carbon were standardized according to the amount of polysaccharide adsorbed, 35 mg. SSS Pneumococcus I 61002-1 per gm. of carbon and 22.5 mg. SSS Pneumococcus III 61153 per gm. of carbon, so that they contained per cc. specific carbohydrate equivalent to a certain dilution of the polysaccharide in solution.

Bacterial Polysaccharides.—Various preparations of specific carbohydrate from Pneumococcus Type I, II and III of varying purity were used in the course of study. Polysaccharides of Pneumococcus Type I, II and III were kindly supplied by Dr. M. Heidelberger and will be designated as SSS Pneumococcus I H, SSS Pneumococcus II H and SSS Pneumococcus III H.

The polysaccharides of Pneumococcus Type I designated SSS Pneumococcus I M and No. 61002-1 were prepared by us according to a method previously described by Heidelberger, Sia and Kendall (10). The polysaccharide of Pneumococcus Type I designated SSS Pneumococcus I F and that of Pneumococcus Type II designated SSS Pneumococcus II G were prepared according to the same method with the exception that the precipitation at the isoelectric point was not carried out, the only purification being that of repeated reprecipitation with alcohol. The polysaccharide of Pneumococcus Type III, No. 61153, was prepared according to the method of Heidelberger and Avery (11) for the preparation of the nitrogen-free specific carbohydrate of Pneumococcus Type III.

None of these polysaccharides were obtained in sufficient amount for chemical analysis but all precipitated specifically their homologous serums in dilutions of 1:5,000,000 to 1:6,000,000.

Immunization of Animals.—Mice, 17 to 19 gm. in weight, were injected by the intraperitoneal route unless otherwise mentioned. Either a single injection was employed or 1 injection weekly for a number of weeks as stated.

Protection Tests.—Animals were tested for immunity by the intraperitoneal injection in serial dilutions, of young cultures of Pneumococcus Type I, II and III of maximum virulence. The virulence of the culture was controlled by simultaneous injection into normal untreated mice. Immunity was considered as demonstrated if the mouse survived the injection of ten or more minimal fatal doses for at least 4 days.

Immunization with Collodion Particles Adsorbed with Pneumococcus Type I, II and III Polysaccharides

Type I.—A washed suspension of collodion particles adsorbed with Pneumococcus Type I polysaccharide M, was injected into mice by various routes, intravenously, intraperitoneally and subcutaneously. One group received 0.5 cc. of a 25,000 million suspension by each route, the other 0.5 cc. of a 50,000 million suspension by the same routes. In

TABLE I

Influence of Route and Quantity on the Immunization of Mice with Collodion Particles Adsorbed with Type I Pneumococcus Polysaccharide M

Dose of collodion particles SSS Pneumococcus I M, measured by density of suspension	Dose of Pneumococcus I culture received 7 days later	Route of immunizing injection								
		Intravenously			Intraperitoneally			Subcutaneously		
	cc.									
0	10^{-9}	S	S	—						
(normal control	10^{-8}	36	36	—						
mice)	10^{-7}	36	36	—						
12,500 $\overline{M}$	10^{-6}	96	S	S	S	S	S	S	S	S
	10^{-5}	40	72	S	72	S	S	S	S	S
	10^{-4}	S	S	S	36	96	S	24	S	S
25,000 $\overline{M}$	10^{-6}	S	S	S	S	S	S	S	S	S
	10^{-5}	S	S	S	36	S	—	96	S	S
	10^{-4}	36	36	S	36	S	S	S	S	—

S = survived.
Numeral = died, hours.
— = not done.

the results as shown in Table I one can notice that the degree of active immunity induced, irrespective of the route of injection, was sufficient to withstand the injection of 1,000 to 10,000 M.F.D. No greater immunity was produced by the use of a double strength suspension.

The intraperitoneal route and a dose of 0.5 cc. of a 25,000 million suspension of adsorbed collodion particles was adopted as our standard in the further immunological study.

In order to determine the time interval required for development of

immunity following the injection of collodion particles adsorbed with Pneumococcus Type I polysaccharide, groups of mice were injected with collodion particles SSS Pneumococcus I M and tested after various intervals for immunity against virulent Pneumococcus Type I culture. Definite immunity was found to have developed within 4 days, in-

TABLE II

Time Factor in the Immunization of Mice with Collodion Particles Adsorbed with Pneumococcus Type I Polysaccharide

Amount of collodion particles SSS Pneumococcus I, measured by density of suspension	Pneumococcus Type I culture dose	Interval between last immunizing injection and test for immunity																		
		3 days			4 days			1 wk.			2 wks.			3 wks.			4 wks.		
	cc.																			
0	10^{-9}	S	S	—	S	S	—	S	S	—	S	S	—	48	48	—	S	S	—
(normal control	10^{-8}	S	S	—	48	S	—	36	36	—	24	36	—	48	48	—	48	48	—
mice)	10^{-7}	48	48	—	36	36	—	36	36	—	36	36	—	48	48	—	48	48	—
12,500 $\overline{M}$	10^{-6}	48	48	48	S	S	S	36	S	S	36	S	S	36	S	S	S	S	—
	10^{-5}	24	48	48	72	S	S	36	S	S	22	S	S	36	S	S	48	96	—
	10^{-4}	24	48	48	48	48	96	36	36	36	S	S	S	24	24	36	48	48	—
	10^{-3}	—	—	—	48	48	48	24	72	S	22	72	S	18	18	24	—	—	—
2 injections of 12,500	10^{-5}							S	S	S	36	S	S	96	S	S	S	S	S
$\overline{M}$ at weekly in-	10^{-4}							S	S	S	18	S	S	S	S	S	36	36	S
tervals	10^{-3}							18	22	S	18	24	S	18	48	S	18	20	—
	10^{-2}							—	—	—	18	18	S	—	—	—	—	—	—
3 injections of 12,500	10^{-5}							S	S	S	96	S	S	S	S	S	36	S	S
$\overline{M}$ at weekly in-	10^{-4}							96	S	S	S	S	S	S	S	S	S	S	S
tervals	10^{-3}							18	24	24	96	S	S	36	S	S	18	36	36
	10^{-2}							18	18	24	—	—	—	—	—	—	18	18	—

S = survived.
Numeral = died, hours.
— = not done.

creasing by the 7th day and reaching its height in about 2 weeks (Table II). Following 2 or 3 weekly injections this immunity is more regular and more marked for a longer period after the final injection. In all groups, however, a decrease in immunity was shown after an interval of 4 weeks.

The specificity of the immunity induced by the injection of collodion

particles adsorbed with Pneumococcus Type I polysaccharide was demonstrated by dividing into three groups a series of mice injected 7 days before with collodion particles SSS Pneumococcus I M. One group then received live virulent cultures of Pneumococcus Type I, another of Type II and the third of Type III. The mice were protected against a dose of the Pneumococcus Type I culture equivalent to 100,000 M.F.D. but showed no immunity against the Type II or Type III organisms as shown in Table III.

TABLE III

Type Specificity in the Immunization of Mice with Collodion Particles Adsorbed with Pneumococcus Type I Polysaccharide

Amount of collodion particles SSS Pneumococcus I M received 7 days before, measured by density of suspension	Pneumococcus Type I				Pneumococcus Type II				Pneumococcus Type III			
	cc.				*cc.*				*cc.*			
0	10^{-9}	36	S	—	10^{-9}	48	48	—	10^{-9}	36	S	—
(normal control	10^{-8}	36	36	—	10^{-8}	48	48	—	10^{-8}	36	72	—
mice)	10^{-7}	36	36	—	10^{-7}	24	48	—	10^{-7}	36	S	—
12,500 $\overline{M}$	10^{-5}	36	S	S	10^{-7}	24	48	48	10^{-7}	36	36	36
	10^{-4}	36	36	S	10^{-6}	24	24	48	10^{-6}	36	36	36
	10^{-3}	20	S	S	10^{-5}	24	24	48	10^{-5}	20	36	—

S = survived.
Numeral = died, hours.
— = not done.

These findings closely correspond with those in mice following a single intraperitoneal injection of formalin-killed Pneumococcus Type I organisms both in the time interval necessary (3 to 7 days) and the specificity. The optimum dosage of 0.5 cc. of a suspension of 100 million organisms per cc. for this single injection is of special interest in view of later findings.

Type II.—Collodion particles adsorbed with Pneumococcus Type II polysaccharide G and washed five times, were injected intraperitoneally into mice which were tested 7 days after the final injection for immunity against live virulent Pneumococcus Type II. After a

TABLE IV

Immunization of Mice with Collodion Particles Adsorbed with Pneumococcus Type II

Amount of collodion particles SSS Pneumococcus II G received, measured by density of suspension	Dose Pneumococcus II culture received 7 days after last injection	No. of immunizing doses received at weekly intervals																											
		1								2								3								4			
	cc.																												
0	10^{-9}	24		S		S		S		36		72		72		27		24		S		S		S		36		72		S		S	
(normal control	10^{-8}	24		36		48		S		36		36		36		36		24		24		48		48		24		24		24		48	
mice)	10^{-7}	36		48		48		72		24		36		36		36		24		24		48		48		24		24		24		48	
12,500 $\overline{M}$ in each	10^{-8}	36	36	48	48	S	S	S	S	24	36	36	72	S	S	S	—	S	S	S	—	—	—	—	—								
dose	10^{-7}	24	36	36	36	36	S	S	S	36	36	72	S	S	S	S	S	36	36	S	S	S	S	S	S	S	S	S	—	—	—	—		
	10^{-6}	24	36	36	48	48	48	S	S	36	36	36	36	36	72	S	S	36	48	S	S	S	S	S	S	24	S	S	S	S	S	S	S	S
	10^{-5}	24	36	36	—	—	—	—	—	36	72	72	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	10^{-4}																	36	48	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	10^{-3}																									S	S	S	S	—	—	—	—		
	10^{-2}																									36	72	S	—	—	—	—	—		

S = survived.
Numeral = died, hours.
— = not done.

single injection immunity to 10 M.F.D. of Pneumococcus Type II was demonstrated. Following 2, 3 or 4 injections at weekly intervals this increased to a protection against 1,000 to 1,000,000 M.F.D. (Table IV). Such immunity as induced by 1 injection of collodion particles SSS II G is specific, no protection being shown against Pneumococcus Type I (Table V).

Collodion particles adsorbed with pneumococcus polysaccharide Type II H, however, produced no demonstrable immunity in mice.

TABLE V

Type Specificity in the Immunization of Mice with Collodion Particles Adsorbed with Pneumococcus Type II Polysaccharide

Amount of collodion particles SSS Pneumococcus II G received 7 days before, measured by density of suspension	Pneumococcus Type I							Pneumococcus Type II						
	cc.							*cc.*						
0	10^{-9}	48	S	S	—	—	—	10^{-9}	S	S	—	—	—	—
(normal control	10^{-8}	48	48	48	—	—	—	10^{-8}	48	48	—	—	—	—
mice)	10^{-7}	48	48	48	—	—	—	10^{-7}	48	48	—	—	—	—
12,500 $\overline{M}$	10^{-8}	48	48	48	72	72	S	10^{-8}	48	S	S	S	S	S
	10^{-7}	48	48	48	48	72	S	10^{-7}	48	48	48	48	S	S
	10^{-6}	48	48	48	48	48	48	10^{-6}	20	48	48	48	48	48
	10^{-5}	48	48	48	48	48	—	10^{-5}	20	24	48	48	S	S

S = survived.
Numeral = died, hours.
— = not done.

This will be discussed further in connection with the immunization with the various polysaccharides in solutions.

Type III.—Collodion particles adsorbed with pneumococcus polysaccharide Type III H also produced no immunity against live virulent organisms of the homologous type. A definite immunity was demonstrable against the heterologous Type I culture but none against Type II (Table VI).

TABLE VI

Immunization of Mice with Collodion Particles Adsorbed with Pneumococcus Type III Polysaccharide

Dose of collodion particles SSS Pneumococcus III H received each injection, measured in density of suspension	Dose of culture received 7 days after last injection	No. of weekly immunizing injections received											
		1			2			3			4		
		Pneumococcus Type I											
	cc.												
0	10^{-9}	48	S		48	S		48	72		48	72	
(normal control	10^{-8}	48	48		48	48		48	48		48	48	
mice)	10^{-7}	48	48		48	48		48	48		48	48	
12,500 $\overline{M}$	10^{-8}	48	S	S	48	48	S						
	10^{-7}	48	—	—	S	S	—	S	S		S	—	
	10^{-6}							S	S		S	—	
	10^{-5}							S	S				
	10^{-4}							S	S				
		Pneumococcus Type II											
0	10^{-9}	S	S		48	48	—	48	S	—			
(normal control	10^{-8}	48	48		48	48	—	24	24	—			
mice)	10^{-7}	48	48		48	48	—	24	48	—			
12,500 $\overline{M}$	10^{-8}	48	48	48	48	48	48	48	48	—	48	48	—
	10^{-7}	48	48	48	48	48	S	24	48	—	—	—	—
		Pneumococcus Type III											
0	10^{-9}	48	S		48	S	—	S	S		S	S	
(normal control	10^{-8}	24	48		48	48	—	S	S		S	S	
mice)	10^{-7}	24	24		48	48	—	72	S		72	S	
12,500 $\overline{M}$	10^{-8}	24	48	S	48	48	S	S	S	—	S	S	—
	10^{-7}	24	48	48	24	24	24	48	S	—			
	10^{-6}	24	48	48	48	S	—	48	96	—			

S = survived.

Numeral = died, hours.

— Not done; died in course of immunization.

The Effect of Free Polysaccharide in Unwashed Suspensions of Collodion Particles Adsorbed with Pneumococcus Type I and II Polysaccharide on the Production of Active Immunity in Mice

Since control mice injected with 1:1,000 or 1:10,000 solutions of pneumococcus polysaccharides Type I, II and III had failed to show immunity (8) the immunity induced by the injection of collodion particles adsorbed with Pneumococcus Type I and II polysaccharide was attributed to the polysaccharide-colloid combination. The minute amount of polysaccharide necessary for the production of immunity was demonstrated by calculating the approximate amount of polysaccharide adsorbed on the collodion particle.

It has now been found that if animals are injected with collodion particles adsorbed with pneumococcus polysaccharide Type I or Type II and not washed free of unadsorbed polysaccharide a very different result will be obtained. No immunity was induced by the injection of unwashed collodion particles SSS Pneumococcus I M or collodion particles SSS II G, whereas a slight immunity was demonstrated following the injection of the same collodion particles washed free of unadsorbed polysaccharide.

The optimum concentration for immunization of polysaccharide solution mixed with collodion particles for adsorption, the excess not being removed, was determined for both Type I and Type II pneumococcus polysaccharides. Whereas in the regular preparation of adsorbed collodion particles these were exposed to a 1:100 solution of the polysaccharide, in this series they were suspended in the same density (25,000,000,000 per cc.) in dilution of the polysaccharide ranging from a 1:1,000 to a 1:5,000,000. The 1:1,000,000 and 1:5,000,000 SSS Pneumococcus I M collodion particles were found to be as effective immunizing antigens as the washed collodion particles SSS Pneumococcus I M and superior to the collodion particles in the stronger dilutions (Table VII).

Immunization with Pneumococcus Type I, II and III Polysaccharide Solutions

The results of these tests led us to investigate our previous failure to induce immunity against Pneumococcus Type I or II by the

TABLE VII

Immunization of Mice with Collodion Particles Adsorbed with Type I Pneumococcus Polysaccharides; Optimum Concentration of Polysaccharides, Free Excess Not Being Removed

Collodion particles 12,500 $\overline{M}$ in dilution of SSS Pneumococcus I M of	Dose of Pneumococcus I culture received 7 days later							Collodion particles 12,500 $\overline{M}$ in dilution of SSS Pneumococcus I M of	Dose of Pneumococcus I culture received 7 days later						
	cc.								*cc.*						
0	10^{-9}	48	S	S	—	—	—	1:500,000 dilution	10^{-8}	96	S	S	S	S	S
(normal control	10^{-8}	48	48	48	—	—	—		10^{-7}	72	96	S	S	S	S
mice)	10^{-7}	48	48	48	—	—	—		10^{-6}	48	48	48	48	S	S
									10^{-5}	48	48	48	72	96	S
1:1,000 dilution	10^{-8}	18	48	48	48	S	S		10^{-4}	48	48	48	48	48	48
	10^{-7}	48	48	48	48	48	48		10^{-3}	18	18	48	48	72	S
	10^{-6}	48	48	48	48	S	S								
	10^{-5}	48	48	48	48	48	S	1:1,000,000 dilution	10^{-8}	S	S	S	S	S	S
	10^{-4}	48	48	48	48	48	48		10^{-7}	96	S	S	S	S	S
	10^{-3}	18	18	24	48	48	48		10^{-6}	48	72	S	S	S	S
									10^{-5}	48	72	96	S	S	S
1:10,000 dilution	10^{-8}	48	48	72	72	S	S		10^{-4}	48	72	96	S	S	S
	10^{-7}	48	48	48	S	S	S		10^{-3}	48	48	48	48	48	48
	10^{-6}	48	48	48	72	S	S								
	10^{-5}	48	48	48	48	48	S	1:5,000,000 dilution	10^{-8}	48	S	S	S	S	S
	10^{-4}	20	24	48	48	48	48		10^{-7}	48	96	96	S	S	S
	10^{-3}	18	18	20	48	48	48		10^{-6}	48	48	96	S	S	S
									10^{-5}	48	48	S	S	S	S
1:50,000 dilution	10^{-8}	48	48	48	48	S	S		10^{-4}	48	48	48	48	72	S
	10^{-7}	48	48	S	S	S	S		10^{-3}	48	48	48	48	48	48
	10^{-6}	48	48	72	S	S	S								
	10^{-5}	48	48	48	S	S	S	Dose washed collodion particles SSS Pneumococcus I M, measured in density of suspension	Dose of Pneumococcus I culture received 7 days later						
	10^{-4}	48	48	48	48	48	S								
	10^{-3}	20	48	48	48	48	48		*cc.*						
1:100,000 dilution	10^{-8}	48	72	72	S	S	S								
	10^{-7}	48	48	96	S	S	S								
	10^{-6}	48	48	48	48	S	S	12,500 $\overline{M}$	10^{-8}	48	48	S	S	S	S
	10^{-5}	48	48	48	48	72	72		10^{-7}	48	48	S	S	S	S
	10^{-4}	24	48	48	48	S	S		10^{-6}	S	S	S	S	S	S
	10^{-3}	18	18	48	48	48	48		10^{-5}	48	48	48	S	S	S
									10^{-4}	48	48	S	S	S	S
									10^{-3}	18	48	48	—	—	—

S = survived.
Numeral = died, hours.
— = not done.

TABLE VIII

Immunization of Mice with Pneumococcus Type I Polysaccharide Solution. Comparison of Polysaccharide Preparations as Immunizing Antigens

Dose of Pneumococcus I polysaccharides	Dose of Pneumococcus I culture received 7 days later	SSS Pneumococcus I M					SSS Pneumococcus I F					SSS Pneumococcus I H												
	cc.																							
0	10^{-9}	36	36	36	S	S	S	S	S	S	S													
(normal control mice)	10^{-8}	36	36	36	36	36	36	36	36	36	48													
	10^{-7}	24	24	24	36	36	36	36	36	36	48													
1:150,000 dilution	10^{-8}	36	S	S	—	—	36	36	36	S	S	36	36	36	36	36	36	72	S	S	S	S	S	S	S
	10^{-7}	36	36	96	S	S	96	S	S	S	S	36	36	36	36	36	36	36	36	36	96	96	S	S	S
	10^{-6}	36	36	96	S	S	36	36	36	S	S	24	24	24	36	36	36	36	36	36	36	36	S	S	S
	10^{-5}	36	36	S	S	S	20	36	36	S	S	24	24	24	36	36	36	36	36	36	36	36	S	S	S
	10^{-4}	20	36	36	36	36	36	36	36	36	S	24	24	24	24	24	24	24	36	36	36	36	36	36	—
1:1,500,000 dilution	10^{-8}	—	—	—	—	—	S	S	S	S	S	36	36	36	72	S	S	S	S	S	S	S	S	S	S
	10^{-7}	—	—	—	—	—	36	42	S	S	S	36	36	36	36	36	72	96	S	S	S	S	S	S	S
	10^{-6}	—	—	—	—	—	36	S	S	S	S	36	36	36	36	36	36	36	48	S	S	S	S	S	S
	10^{-5}	—	—	—	—	—	36	36	36	36	S	24	24	24	36	36	36	36	36	36	S	S	S	S	S
	10^{-4}	—	—	—	—	—	36	36	36	36	36	24	24	24	24	24	24	36	36	36	S	S	S	S	S
1:3,000,000 dilution	10^{-8}	S	S	S	—	—	S	S	S	S	S	36	36	36	48	S	S	S	S	S	S	S	S	S	S
	10^{-7}	96	S	S	—	—	S	S	S	S	S	36	36	48	96	96	S	S	S	S	S	S	S	S	S
	10^{-6}	S	S	S	—	—	96	S	S	S	S	36	36	36	36	72	72	S	S	S	S	S	S	S	S
	10^{-5}	S	96	96	—	—	96	S	S	S	S	36	36	36	48	96	S	S	S	S	S	S	S	S	S
	10^{-4}	36	36	36	—	—	24	24	S	S	S	24	24	24	36	36	36	36	36	36	36	36	72	S	S
1:6,000,000 dilution	10^{-8}	96	S	S	—	—	72	S	S	S	S	36	48	72	72	S	S	S	S	S	S	S	S	S	S
	10^{-7}	S	S	S	—	—	72	S	S	S	S	36	36	96	S	S	S	S	S	S	S	S	S	S	S
	10^{-6}	36	36	S	—	—	72	S	S	S	S	36	36	48	72	96	96	S	S	S	S	S	S	S	S
	10^{-5}	36	S	72	—	—	72	96	S	S	S	36	36	36	36	36	36	36	72	S	S	S	S	S	S
	10^{-4}	36	36	36	—	—	18	24	36	S	—	20	24	24	24	24	24	24	24	24	36	36	36	S	—	—

S = survived.
Numeral = died, hours.
— = not done.

injection of the homologous specific polysaccharide. The dilutions of polysaccharide previously used were 1:1,000 and 1:10,000; and

while an occasional survival was noted among the injected mice when tested with live virulent pneumococci these were too irregular to indicate any definite immunity in the groups.

Pneumococcus Type I Polysaccharide.—A preliminary test showed that although SSS Pneumococcus I M, the polysaccharide used for adsorption on the collodion particles in the preceding studies, produced no immunity when injected alone into mice in a 1:10,000 dilution, a 1:150,000 dilution produced very definite immunity. Similar results were obtained by Schiemann, Wadsworth and Brown, Saito and Ulrich and Enders but, as mentioned before, by the use of specific carbohydrate substances derived from the disrupted pneumococcus cell and assumed to be distinct from the specific soluble carbohydrate of Heidelberger and Avery.

The comparative test shown in Table VIII was therefore carried out with the three Pneumococcus Type I polysaccharides already described. The dilution which produced highest immunity was found to be 1:3,000,000. A protection against 100 to 10,000 M.F.D. was obtained by a single injection of 1 cc. of this dilution. The variations observed in the immunity produced by the polysaccharides are probably due to difference in purity. These polysaccharides were found by adsorption tests to absorb from monovalent Type I serum precipitins against each. After boiling for 1 hour at pH 9 their activity was retained both as shown by the precipitation reaction with homologous serum and by the production of immunity in mice. These polysaccharides differ, therefore, from both the A substance of Enders and the antigenic carbohydrate of Wadsworth and Brown. The immunity produced by 1, 2 or 3 weekly doses of the least pure of these polysaccharides, SSS Pneumococcus I F, proved to be specific for Pneumococcus Type I. An immunity was obtained against 10,000 M.F.D. of Type I pneumococcus culture after three weekly doses while no immunity could be demonstrated against live Pneumococcus Type II or Type III.

The time interval necessary for production of immunity by this polysaccharide solution was investigated—a slight immunity is shown 4 days after injection, reaching its height in 7 days and beginning to decrease after 2 weeks, though still demonstrable after 4 weeks.

Keeping in mind the aggressin-like action noted with higher con-

centrations of the pneumococcus polysaccharide and described also by Felton (12) and Sia (13, 14) we gave to a group of mice an injection of a polysaccharide solution of 1:10,000 dilution 1 week after the initial immunizing injection. By protection tests we found the resistance

TABLE IX

Optimum Dilution in the Immunization of Mice with Pneumococcus Polysaccharide Type II

Dose of SSS Pneumococcus II G 1 cc. of	Dose of Pneumococcus II culture received 9 days later					
	cc.					
0	10^{-9}	24	36			
(normal control	10^{-8}	36	36			
mice)	10^{-7}	20	36			
1:200,000 dilution	10^{-8}	48	48	48	48	48
	10^{-7}	24	48	48	S	S
	10^{-6}	48	48	48	48	48
	10^{-5}	20	24	24	48	72
1:500,000 dilution	10^{-8}	48	48	48	S	S
	10^{-7}	24	48	48	48	S
	10^{-6}	20	24	48	48	48
	10^{-5}	24	24	48	48	72
1:1,000,000 dilution	10^{-8}	48	S	S	S	S
	10^{-7}	48	48	S	S	S
	10^{-6}	48	48	S	S	S
	10^{-5}	24	48	72	S	S
1:5,000,000 dilution	10^{-8}	48	48	96	S	S
	10^{-7}	S	S	S	S	S
	10^{-6}	24	24	72	S	S
	10^{-5}	20	24	48	S	S

S = survived.
Numeral = died, hours.

was lowered from that effective against 10,000 M.F.D. to one effective against 10 to 100 M.F.D.

Pneumococcus Type II Polysaccharide.—Solutions of Pneumococcus Type II polysaccharide SSS Pneumococcus II G (the same poly-

TABLE X

Specificity in the Immunization of Mice with Pneumococcus Type II Polysaccharide

No. of weekly doses of 1 cc. of 1:2,000,000 SSS Pneumococcus II G	Dose of Pneumococcus culture received 7 days after final injections	Pneumococcus Type I					Pneumococcus Type II					Pneumococcus Type III		
	cc.													
0	10^{-9}	98	S				24	S				24	48	
(normal control	10^{-8}	48	48				24	48				48	48	
mice)	10^{-7}	24	48				24	48				24	48	
1	10^{-8}	48	S	S	S	S						24	24	48
	10^{-7}	48	48	S	S	S	24	48	S	S	S	24	24	24
	10^{-6}	48	96	S	S	S	48	48	48	S	S	24	24	48
	10^{-5}						24	24	48	S	S			
	10^{-4}						24	24	24	24	S			
0	10^{-9}	36	S				36	72				S	S	
(normal control	10^{-8}	36	36				24	24				S	S	
mice)	10^{-7}	24	36				24	24				24	S	
2	10^{-8}	36	S	S	S	S						36	S	S
	10^{-7}	36	36	S	S	S	36	36	S	S	S	36	S	S
	10^{-6}	72	72	72	S	S	36	36	72	S	S			
	10^{-5}						24	S	S	S	S			
	10^{-4}						36	36	36	S	S			
	10^{-3}						18	36	36	—	—			
0	10^{-9}	48	S				24	S				48	48	
(normal control	10^{-8}	48	48				24	24				48	48	
mice)	10^{-7}	48	48				24	48				20	24	
3	10^{-8}	48	48	S	—	—						24	24	24
	10^{-7}	48	72	S	S	—	72	S	S	—		20	24	48
	10^{-6}	48	S	S	S	S	24	96	S	S				
	10^{-5}						18	24	48	48	48			
	10^{-4}													

S = survived.
Numeral = died, hours.
— = not done.

saccharide used with adsorbed collodion particles in Table IV), when injected into mice in sufficiently high dilutions, produced a definite immunity as shown in Table IX. The immunity produced

TABLE XI

Immunization of Mice with Pneumococcus Type III Polysaccharide

Dose of SSS Pneumococcus III H received each injection 1 cc. of	Dose of culture received 7 days after last injection	No. of weekly immunizing injections received											
		1			2			3			4		
		Pneumococcus Type I											
	cc.												
0	10^{-9}	48	S		48	S		48	72		48	72	
(normal control	10^{-8}	48	48		48	48		48	48		48	48	
mice)	10^{-7}	48	48		48	48		48	48		48	48	
1:300,000 dilution	10^{-8}	24	S	S	S	S	S						
	10^{-7}	48	S	S	48	S	S	18	S	S	72	96	S
	10^{-6}				S	S	S	S	S	S	S	S	—
	10^{-5}							72	S	S	96	S	—
	10^{-4}							72	S	S	48	S	—
1:3 $\overline{M}$ dilution	10^{-8}	48	48	S	48	48	72	18	48	S	S	S	—
	10^{-7}	48	48	S	48	48	S	48	48	S	48	S	—
					48	S		S	S	S	S	S	—
		Pneumococcus Type II											
0	10^{-9}	S	S		48	48	—	48	S	—	48	S	
(normal control	10^{-8}	48	48		48	48	—	24	24	—	24	24	
mice)	10^{-7}	48	48		48	48	—	24	48	—	24	48	
1:300,000	10^{-8}	48	48	S	48	48	48	24	S	—	48	S	—
	10^{-7}	48	48	48	48	72	72	24	S	—	S	S	—
1:3 $\overline{M}$ dilution	10^{-8}	48	48	48	48	48	48	48	72	—	S	S	—
	10^{-7}	48	48	48	48	48	48	24	48	—	24	—	—
		Pneumococcus Type III											
0	10^{-9}	48	S		48	S	—	S	S		S	S	
(normal control	10^{-8}	24	48		48	48	—	S	S		S	S	
mice)	10^{-7}	24	24		48	48	—	72	S		72	S	
1:300,000 dilution	10^{-8}	48	48	48	48	48	48	S	S	—	S	S	
	10^{-7}	24	48	48	48	48	48	48	S	—	48	S	
	10^{-6}	24	24	48	—	—	—	—	—	—			
1:3 $\overline{M}$ dilution	10^{-8}	24	48	48	24	24	48	96	S	S	S	—	—
	10^{-7}	24	48	48	18	24	24	24	48	48	S	—	—
	10^{-6}	48	48	48	—	—	—						

S = survived.

Numeral = died, hours.

— = not done, died in course of immunization.

by this not sufficiently purified polysaccharide was found, however, to be non-specific, protecting against Pneumococcus Type I culture as well as Type II though not against Type III (Table X). However, as in the experience of Avery and his coworkers (1, 2), SSS Pneumo-

TABLE XII

Immunization of Mice with Carbon Adsorbed with Type III Pneumococcus Polysaccharide 61153 and Solutions of Type III Pneumococcus Polysaccharide 61153

Dose of SSS Pneumococcus III 61153 0.5 cc. injected weekly for 4 wks.	Dose of Pneumococcus III culture injected 7 days after final injection	Immunizing dose adsorbed on carbon*					Immunizing dose in solution				
	cc.										
1:10,000 dilution	10^{-8}	48	48	48	48	48	48	48	48	48	48
	10^{-7}	24	48	48	48	48	24	24	48	48	48
1:100,000 dilution	10^{-8}	48	48	48	48		24	48	48	48	48
	10^{-7}	48	48	48	S	S	48	48	48	48	48
	10^{-6}	24	24	24	S	S					
1:1,000,000 dilution	10^{-8}	48	S	S	S	S	48	48	48	48	—
	10^{-7}	24	48	48	72	S	24	48	48	48	—
0	10^{-9}	48	S	—							
(normal control)	10^{-8}	48	48	—							
	10^{-7}	48	48	—							
Carbon 44.4 mg. per cc.	10^{-8}	48	48	48	48	48					
	10^{-7}	24	48	48	—	—					

S = survived.

Normal = died, hours.

— = not done.

* 1 gm. carbon adsorbed 22.5 mg. Pneumococcus III SSS 61153.

1 gm. carbon adsorbed with Pneumococcus III SSS 61153, in 225 cc. saline contains per cc. 44.4 mg. of carbon adsorbed with 0.1 mg. of Pneumococcus III SSS or 1 part polysaccharide in 1,000.

coccus II H, the purified carbohydrate received from Dr. Heidelberger, when injected in solution or adsorbed on collodion particles, produced no immunity against pneumococci of any type.

Pneumococcus Type III Polysaccharide.—A solution of Pneumo-

coccus Type III polysaccharide SSS Pneumococcus III H when injected into mice produced no definite immunity against the homologous live culture. However, as was the case when using this polysaccharide adsorbed on collodion particles, a definite immunity was demonstrable against live Pneumococcus Type I culture but none against the Type II strain (Table XI).

Immunization with Carbon Adsorbed with Pneumococcus Type I and III Polysaccharides

Washed suspensions of carbon adsorbed with Pneumococcus Type I polysaccharide 61002 and solutions of the same polysaccharide, containing an amount of the specific carbohydrate per cc. equivalent to the adsorbed carbohydrate content per cc. in the carbon suspensions, were injected into a group of mice. After 4 weekly injections tests were made for active immunity against live virulent Pneumococcus Type I. No definite immunity was induced by the polysaccharide in solution but an immunity to 1,000 M.F.D. was found in the mice injected with carbon with 1:100,000 polysaccharide adsorbed upon it.

The same procedure was followed using a Pneumococcus Type III polysaccharide 61153 adsorbed on carbon and unadsorbed. Again the polysaccharide alone produced no immunity against the homologous virulent organism, as had been the case with other preparations of Pneumococcus Type III polysaccharide. However, a slight immunity was observed in the mice injected with the polysaccharide adsorbed on carbon in the higher dilutions (Table XII).

DISCUSSION

Two groups of workers have attempted immunization with the specific polysaccharides of the pneumococcus. The first includes those who have prepared the carbohydrates by the original method of Avery and Heidelberger and the second those who have used other methods of preparation involving especially the use of the whole organism. The latter no doubt obtained a different substance from the one originally reported. With the exception of the human tests reported by Tillett and Francis, by Finland and Sutliff and by ourselves (15), all attempts to immunize animals by the first group of workers have been negative. The tests used as indicative

of antibody response were mainly those for the presence of agglutinins and precipitins. Of the second group of workers, Schiemann and his coworkers, Enders and Wadsworth and Brown have reported successful active immunization both in mice and in rabbits. The special feature of their method of immunization has been the very small dosages. A characteristic property of the polysaccharides used was that the immunizing value was lost when they were boiled in a weak alkaline solution.

In the work here presented some of the polysaccharides used were either prepared by the original method or supplied through the kindness of Dr. M. Heidelberger. All of our specific carbohydrates resisted boiling in weak alkaline solution and some of them had been in solution for over a year prior to their use for immunization.

The principal considerations in our work were the relation of dosages to antibody response, the comparison of antibody response induced by the polysaccharides in solution, adsorbed on collodion particles and on carbon, and the utility of the protection test for active immunity in the detection of this antibody response. We observed marked difference in the antigenicity of the various polysaccharides. The only basis on which we can explain this is that some of the carbohydrates have lost their immunizing property in the process of purification while retaining their serological activity. This explanation is, of course, only tentative.

An interesting phenomenon observed was the cross-immunity against Pneumococcus Type I obtained with Type III polysaccharide, while very little or no immunity was found against Type III. A similar cross-immunity to Pneumococcus Type I was produced by the Type II polysaccharide Lot G, as well as an immunity to the homologous Type II organism. In contrast to this was the lack of immunizing value of the Type II polysaccharide obtained from Dr. Heidelberger. The possible contamination with C substance of polysaccharide G could not account for this crossing, as we found no active immunity to any of the type-specific pneumococci Type I, II or III in response to the injection of either degraded R pneumococci or C substance in various dilutions. The non-antigenicity of the C substance has also been noted by Tillett, Goebel and Avery (16).

The polysaccharides adsorbed on collodion and carbon were more

regularly antigenic. The injection of Type III polysaccharide in various dilutions produced no immunity against the homologous organism, but when it had been adsorbed on carbon a slight immunity was noted. The same was true with one preparation of Type I polysaccharide. These results are interesting in view of the fact that the carbohydrates are irreversibly adsorbed on the carbon. How these can then produce immunity it is difficult to say. Conceivably the carbohydrate is eluted from the carbon in the body or the antibody is formed *in situ* on the particle.

It is difficult to explain the optimum immunizing dose of the free polysaccharide. In large doses it produces an increased susceptibility to infection, in small doses it does not produce immunity. These results suggest a probable capacity of the host to adsorb a limited amount of the carbohydrate, which when injected in larger amounts affects injuriously the special cells concerned in the production of immunity, with the result that the animal becomes more susceptible to infection. This may throw light on the course of events in pneumonia and certain other diseases.

CONCLUSIONS

1. Pneumococcus polysaccharides Types I, II and III adsorbed on collodion particles, and Types I and III adsorbed on carbon (norit) are antigenic in mice.

2. Unadsorbed pneumococcus polysaccharide of Type I is antigenic in mice in proper dilution. One preparation of Type II polysaccharide was not antigenic, while another one immunized against Types I and II. Type III polysaccharide was only slightly antigenic against Type III but immunized against Type I.

3. The antigenicity of pneumococcus polysaccharide in optimal dosage is tentatively explained by an adsorption phenomenon taking place in the body in instances in which the polysaccharides had not been adsorbed before injection.

4. The aggressin-like action of large doses of pneumococcus polysaccharides Types I, II and III is further established.

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